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A HYPERSULPHATED OLIGOSACCHARIDE INHIBITS  
INTRINSIC TENASE AND PROTHROMBINASE,  
KEY COMPONENTS OF THE BLOOD COAGULATION CASCADE

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## DECLARATION

I declare that the research work contributing to this thesis is my own work, and I have indicated the contributions made by others in the text. The thesis has been composed entirely by myself and has not been submitted in candidature for any other degree, diploma or professional qualification.

Signed .

Date 1<sup>st</sup> Oct 1998.



## ABSTRACT

The action of thrombin is central to the processes of thrombosis and haemostasis. Thrombin is generated following the activation of prothrombin by 'prothrombinase' (II-ase), the phospholipid membrane-bound factor Xa (fXa)-factor Va (fVa) complex, which in turn is dependent upon the generation of fXa by intrinsic 'tenase' (X-ase), the phospholipid membrane-bound factor IXa (fIXa)-factor VIIIa (fVIIIa) complex. Thrombin not only converts fibrinogen to fibrin, but also amplifies its own formation by activating the cofactors in II-ase and X-ase, factor V and factor VIII, respectively. The critical role played by II-ase and X-ase towards thrombin generation makes these membrane-bound enzyme complexes attractive targets for inhibition and offers an effective approach to blocking coagulation. Heparin acts as an anticoagulant by activating antithrombin (AT), which then inactivates thrombin, fXa and other activated clotting factors. However, the heparin-AT complex has limited activity against membrane-bound fIXa and fXa. Recently, in buffer systems, heparin has been shown to have an AT-independent effect on coagulation by directly inhibiting X-ase, an effect that is minimal in plasma where the AT-dependent effect predominates (Barrow et al. 1994a). To capitalise on this AT-independent effect, heparin was chemically modified by periodate oxidation and borohydrate reduction to lower its affinity for AT (from  $K_d$  value of 25nM to 43 $\mu$ M); we used LMWH rather than heparin to take advantage of the superior pharmacokinetic profile of LMWH. Using this low affinity LMWH (LA-LMWH), N-desulphated LMWH was prepared using a solvolytic desulphation method. Whereas LA-LMWH inhibited X-ase to a similar extent as LMWH with high AT-affinity ( $IC_{50}$  of 16 $\mu$ g/ml and 13 $\mu$ g/ml respectively), N-desulphated LMWH had minimal inhibitory activity ( $IC_{50}$  of 166 $\mu$ g/ml). These findings indicate that the inhibition of X-ase by LMWH is AT-independent but charge-dependent. To test this concept, LA-LMWH was progressively hypersulphated using sulphur trioxide as a donor. Maximally hypersulphated LMWH (sLMWH) was not only a 32-fold more potent inhibitor of X-ase than LMWH ( $IC_{50}$  reduced from 16 $\mu$ g/ml to 0.5 $\mu$ g/ml), but also acquired II-ase inhibitory activity, with an  $IC_{50}$  of 30 $\mu$ g/ml. In plasma systems, sLMWH had similar AT-independent effects on X-ase and II-ase. Using the activated partial thromboplastin time to reflect the activity of X-ase and II-ase, and the fXa-clotting time to reflect the activity of II-ase, sLMWH prolonged both clotting times in AT-depleted and control plasma to the same extent. In contrast, unfractionated heparin had no effect on either clotting test in AT-depleted plasma. When the effect of sLMWH on various components of X-ase and II-ase was examined, the inhibitory effect was found to be cofactor-dependent (fVa/fVIIIa) and phospholipid-independent. Although assembly and function of II-ase and X-ase on synthetic phospholipid vesicles differs from that on platelets, sLMWH directly inhibited both enzyme complexes on the surface of isolated, washed human platelets. Determination of turnover numbers of both enzyme complexes in the presence of increasing concentrations of sLMWH indicates a differential inhibitory effect, with almost

complete inhibition of fXa generation by X-ase. To further explore the mechanism of inhibition, the Michaelis constant,  $K_m$ , and  $k_{cat}$  of the fXa/fVa complex for prothrombin, and of the fIXa/fVIIIa complex for fX were determined kinetically, in the presence of a saturating concentration of phospholipid (24-50 $\mu$ M), and in the absence or presence of sLMWH. In the presence of 500 $\mu$ g/ml sLMWH, the  $k_{cat}$  was reduced 3-fold for II-ase, whereas the  $K_m$  was unchanged at 0.16 $\mu$ M, consistent with a non-competitive mechanism of inhibition. A similar pattern of inhibition was seen with X-ase; in the presence of 0.5 $\mu$ g/ml sLMWH,  $k_{cat}$  was reduced 4-fold, with minimal changes in  $K_m$ . By acting as a potent direct inhibitor of X-ase and II-ase, sLMWH acts as an anticoagulant in a novel fashion.

## ACKNOWLEDGEMENTS

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*This thesis is dedicated in memory of two very special people,  
my brother, Alan,  
and my father*

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## ABBREVIATIONS

A	absorbance
APTT	activated partial thromboplastin time
ATS	antistasin
AT	antithrombin
COOH	carboxy
CS	chondroitin sulphate
DEGR-CK	dansyl-Glu-Gly-ArgCH <sub>2</sub> Cl
EDTA	ethylenediaminetetra-acetic acid
F1-M-FPR-IXa	fluorescein-thioacetyl-maleimide-FPR-IXa
GAG	glycosaminoglycan
GlcUA	glucuronic acid
GlcN	glucosamine
g	gravitational force
IC <sub>50</sub>	concentration causing inhibition of 50%
IdUA	iduronic acid
k <sub>1</sub>	pseudo first-order rate constant
k <sub>2</sub>	apparent second-order rate constant
Kd <sub>app</sub>	apparent dissociation constant

K <sub>m</sub>	Michaelis constant
k <sub>cat</sub>	turnover number
kDa	kiloDalton
LMWH	low molecular weight heparin
sLMWH	sulphated low molecular weight heparin
PEG	polyethylene glycol
PC	phosphatidylcholine
PS	phosphatidylserine
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAP	tick anticoagulant peptide
TBS	20mM Tris-HCl, pH 7.4, 150mM NaCl
TFPI	tissue factor pathway inhibitor
TMB	Tyrod's modified buffer
UH	unfractionated heparin
II-ase	prothrombinase
X-ase	intrinsic tenase

## **CHAPTER 1 : INTRODUCTION**

### **1.1. The Importance of Intrinsic Tenase and Prothrombinase in Blood Coagulation**

### **1.2. The Surface Membrane Enzyme Complexes, Intrinsic Tenase and Prothrombinase**

#### **1.2.1. The Prothrombinase Complex**

Catalytic Advantage of Prothrombinase Complex Formation

Contribution of Membrane Surface to Enhanced Catalytic Rate of Prothrombinase

Contribution of Activated Cofactor to Enhanced Catalytic Rate of Prothrombinase

#### **1.2.2. The Intrinsic Tenase Complex**

Model of Intrinsic Tenase

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### **1.3. Heparin and Low Molecular Weight Heparin**

#### **1.3.1. Structure of Heparin**

#### **1.3.2. Anticoagulant Effects of Heparin**

Antithrombin-dependent Anticoagulant Effect

Antithrombin-independent Mechanisms of Inhibition

### **1.4. Objectives**

## CHAPTER 1

### INTRODUCTION

Arterial thrombosis is triggered by vascular injury. Spontaneous or traumatic rupture of atherosclerotic plaque exposes tissue factor (TF) which complexes factor VII/VIIa (fVII/VIIa) and triggers coagulation (Davie, 1995) by activating factor IX (fIX) and factor X (fX)(Broze, 1995a). Although dampened by tissue factor pathway inhibitor (TFPI)(Broze, 1995b), sufficient factor Xa (fXa) is generated by TF/VIIa to form thrombin by "prothrombinase", the phospholipid-membrane bound factor Va (fVa)-factor Xa (fXa) complex. In turn, the thrombin generated allows platelet aggregation and activation of the cofactors, factor V (fV) and factor VIII (fVIII). Through the activation of factor IX (fIX), sufficient fXa is generated by "intrinsic tenase", the phospholipid-membrane bound factor VIIIa (fVIIIa)-factor IXa (fIXa) complex, to maintain haemostasis (Broze, 1995a). The critical role of intrinsic tenase and prothrombinase in the coagulation pathway makes these enzyme complexes attractive targets for inhibition, and provides a highly effective approach to block coagulation.

Heparin is a widely used anticoagulant (Hirsh et al., 1995), but has pharmacokinetic and biophysical limitations (Hirsh, 1991)(Eisenberg et al., 1993)(Hogg and Jackson, 1985). The biophysical limitations reflect, in part, the inability of the heparin-antithrombin complex to inactivate fXa bound to platelets within prothrombinase (Marciniak, 1973)(Speijer et al., 1995)(Teitel and Rosenberg, 1983), as well as fIXa within intrinsic tenase.

Heparin acts as an anticoagulant by activating antithrombin (AT), which inactivates

fXa and thrombin (Rosenberg, R. D., 1987). Recently, in buffer systems, heparin and low molecular weight heparin (LMWH) have been shown to have antithrombin-independent effects on blood coagulation by the direct inhibition of intrinsic tenase, but have no inhibitory effect on the function of prothrombinase (Barrow et al., 1994a). In plasma systems, the antithrombin-dependent anticoagulant effect predominates. To capitalise on the antithrombin-independent effect, heparin was chemically modified to reduce its affinity for antithrombin from a  $K_d$  value of 25nM to 43 $\mu$ M (unpublished results) using a technique of periodate oxidation followed by borohydride reduction (Young and Hirsh, 1990). We used LMWH rather than unfractionated heparin to take advantage of the superior pharmacokinetic profile of LMWH (Hirsh and Levine, 1992; Weitz, 1997).

The aim of this study was to explore methods of increasing the potency of LMWH. For this purpose, the low affinity LMWH (LA-LMWH) was N-desulphated by a desulphation solvolytic technique (Inoue and Nagasawa, 1976). Preliminary results indicated that N-desulphated LMWH had minimal inhibitory activity compared to LA-LMWH, suggesting that the inhibition of intrinsic tenase by LMWH was charge-dependent. To test this hypothesis, LA-LMWH was progressively hypersulphated using sulphur trioxide as a sulphate donor and the effect of these hypersulphated LA-LMWH (sLMWH) fractions on intrinsic tenase and prothrombinase was examined.

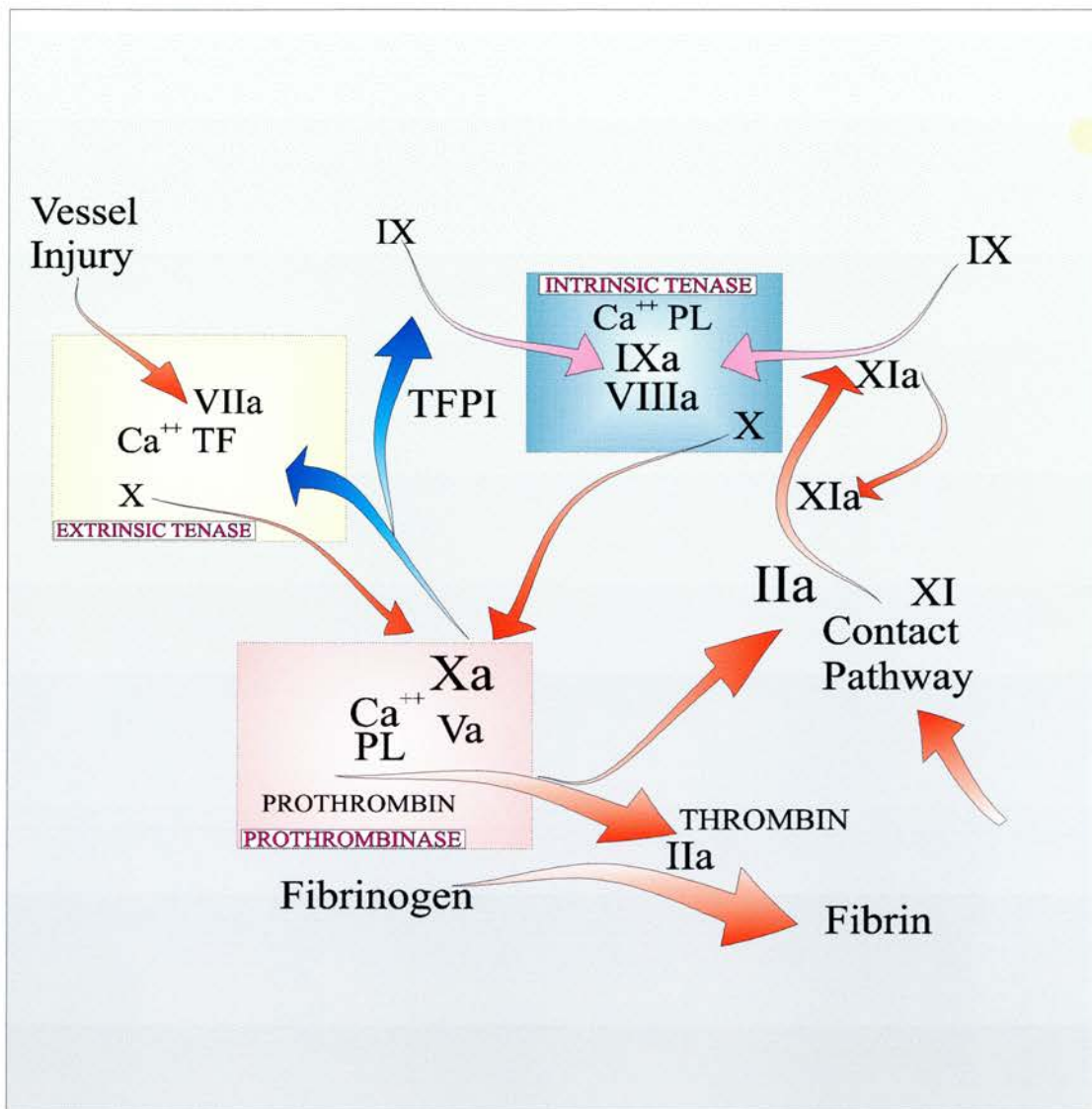
### **1.1 The Importance of Intrinsic Tenase and Prothrombinase in Thrombin Generation :**

The function of the haemostatic mechanism is to maintain blood in its fluid state

and to stop catastrophic blood loss from severed vessels. Maintenance of haemostasis includes interactions between endothelium, platelets and coagulation factors (Nesheim, 1984; Furie and Furie, 1988).

It is currently believed that the haemostatic process does not end with the generation of fXa and thrombin. Instead, the initial haemostatic response must be "consolidated" by the progressive local generation of fXa and thrombin, reflecting the continuous removal of activated cofactors by blood flow, inactivation by protease inhibitors and the competing process of fibrinolysis (Broze, 1995a). In normal haemostasis, the activated fVII/fVIIa-TF complex (extrinsic 'tenase') is responsible for initial fXa generation, and subsequently provides sufficient thrombin by "prothrombinase", the phospholipid membrane bound fXa-fVa complex, to induce local aggregation of platelets and activation of fV and fVIII (Rapaport and Rao, 1995). However, fXa produced by fVIIa/TF is dampened by tissue factor pathway inhibitor (TFPI) (Schneider, 1947; Thomas, 1947; Broze and Miletich, 1987; Broze, 1995a) and is insufficient to sustain haemostasis. TFPI, a multivalent Kunitz-type inhibitor of tissue factor-induced coagulation which directly binds and inhibits fXa, produces feedback inhibition of the fVIIa-TF catalytic complex in a fXa-dependent manner (Broze et al., 1988; Girard et al., 1989; Girard and Broze, 1993). Factor Xa produced by fVIIa/TF must be amplified through the actions of fIXa and fVIIIa within the "intrinsic tenase" complex, the phospholipid membrane bound fIXa-fVIIIa complex, to maintain haemostasis (Broze, 1995a) (see figure 1). As a result of the central roles of intrinsic tenase and prothrombinase, an effective approach to block coagulation would be to





**Figure 1** : Schematic representation of the coagulation cascade, revised in 1995. (Adapted from Broze, G.J., 1995.) Coagulation is initiated at a site of vessel damage following the exposure of fVIIa to tissue factor. As a result of the feedback inhibition of the fVIIa/TF complex by TFPI, factors VIII, IX and XI are required for the production of additional fXa.

directly inhibit the activity of these complexes.

## **1.2. The Surface Membrane Enzyme Complexes, Prothrombinase and Intrinsic Tenase :**

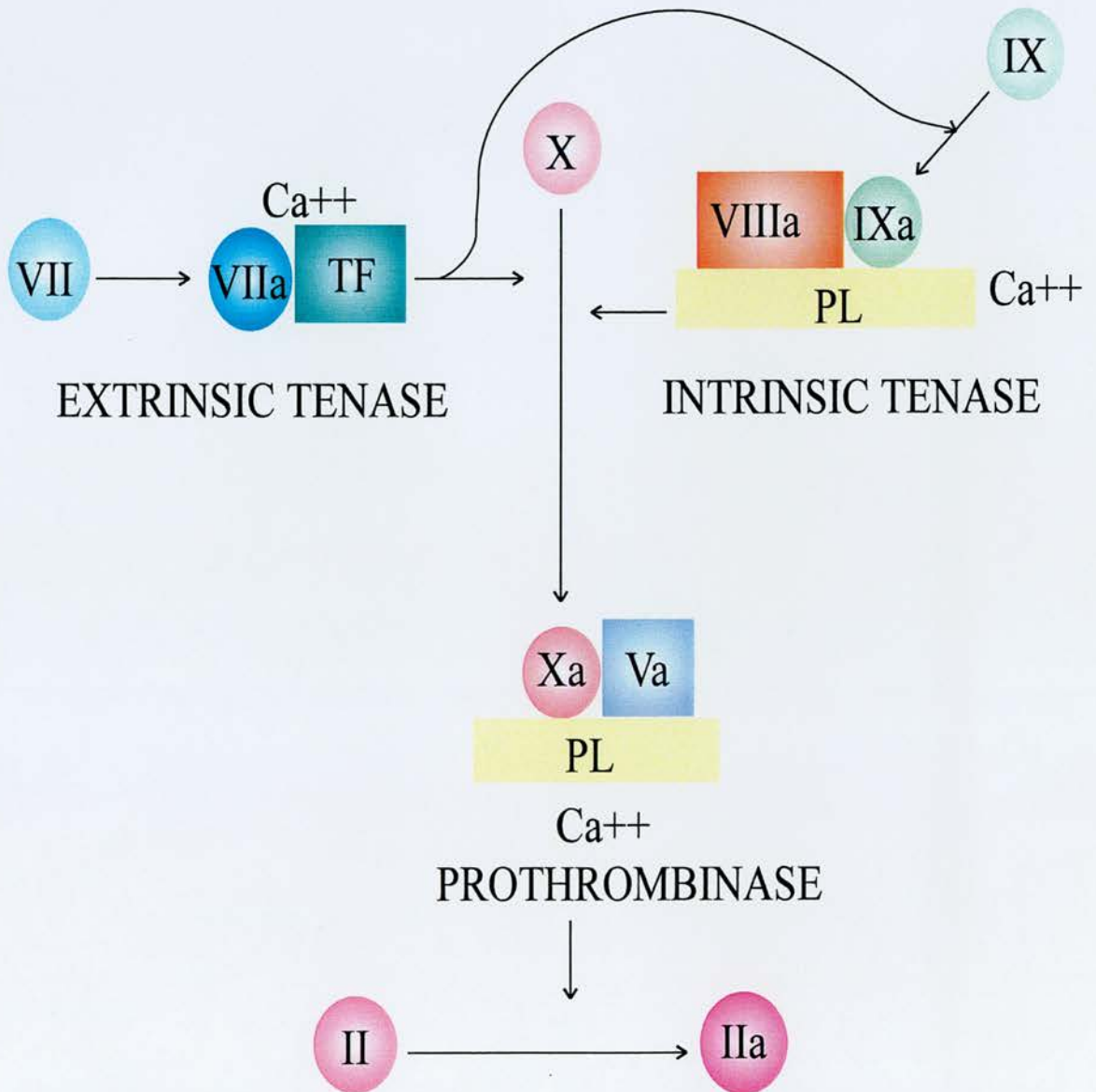
The three prominent enzyme systems that exist within the coagulation pathway are: extrinsic tenase, intrinsic tenase and prothrombinase (see figure 2). Although the extrinsic pathway is responsible for initiation of coagulation, it has a limited role in its propagation. In addition, this system is dependent on components not constitutively present in blood. For these reasons, the extrinsic tenase complex will not be considered further.

Prothrombinase and intrinsic tenase share similar properties. Each complex consists of a vitamin K- dependent serine protease and a non-proteolytic cofactor protein (see figure 3). The reactions are  $\text{Ca}^{2+}$  dependent and require a negatively charged phospholipid surface for the optimal expression of activity within a physiological time scale (Nesheim, 1984)(Mann, 1987)(Mann et al., 1988)(Mann et al., 1990).

### **1.2.1. The Prothrombinase Complex**

The prothrombinase complex is the terminal complex of the blood coagulation cascade and is composed of the serine protease, fXa, associated with the protein cofactor fVa, assembled on a cellular membrane (Krishnaswamy et al., 1987) (Miletich et al., 1978)(Miletich et al., 1978)(Kane et al., 1980)(Tracy et al., 1983) in the presence of calcium ions (Jenny and Mann, 1989)(Mann et al., 1988)(Mann, 1987). The complex can also be assembled in vitro using synthetic phospholipid vesicles (Nesheim et al.,

## ENZYME COMPLEXES OF THE COAGULATION CASCADE

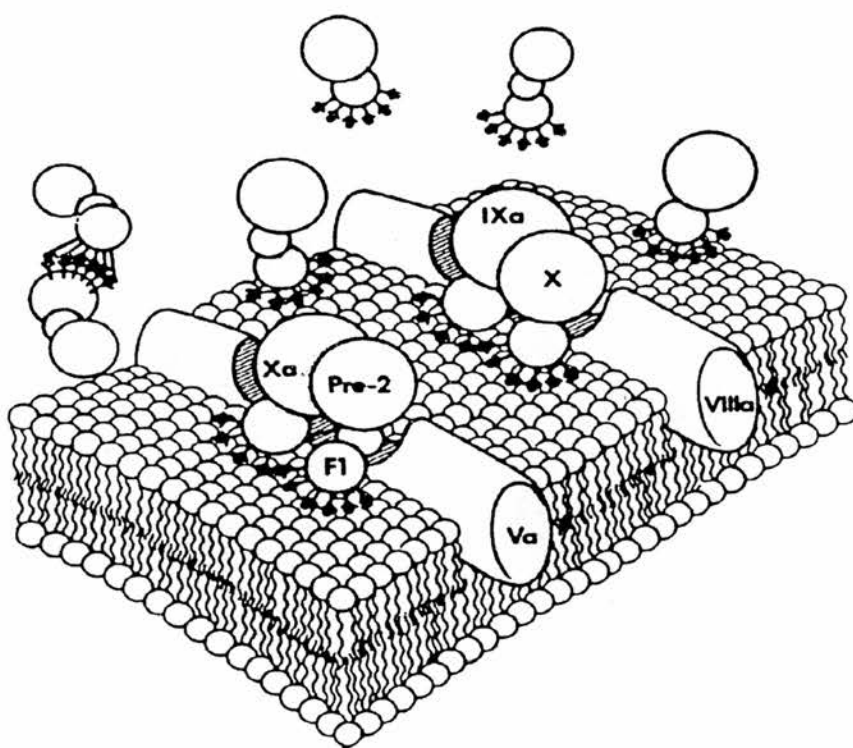


**Figure 2:**

**Schematic diagram showing the Enzyme Complexes of the Coagulation Cascade.**

The prothrombinase and intrinsic tenase complexes share structural and functional similarities; each complex consists of a vitamin K-dependent serine protease and a non-proteolytic cofactor protein. Reactions are calcium-dependent, and also require a negatively charged phospholipid surface.

(Adapted from Mann, 1988)



**Figure 3 :** Diagrammatic representation of the prothrombinase & intrinsic tenase complexes. Structural and functional similarities between the constituents of both complexes suggest that they assemble in a similar manner on a membrane lipid bilayer. (Adapted from Nesheim, 1984).

1979). The complex catalyses the proteolytic activation of prothrombin to thrombin via the proteolysis of two peptide bonds. (Krishnaswamy, 1990)(Krishnaswamy et al., 1993).

Equilibrium binding studies indicate that the prothrombinase complex consists of a phospholipid-bound 1:1 complex of fVa and fXa (Krishnaswamy, 1990) such that fVa constitutes a "receptor" for fXa (Nesheim, 1984)(Tracy and Mann, 1983). Factor Va interacts with the phospholipid membrane via its light chain; fXa, which also interacts with the phospholipid membrane, interacts with the light chain of fVa, and prothrombin interacts with the heavy chain of fVa. The membrane bound complex allows for localisation, amplification and modulation of the reaction (Nesheim, 1984., Tracy and Mann, 1983).

Stopped-flow kinetic studies of prothrombinase assembly have led to the formulation of a model for assembly of the complex (Krishnaswamy et al., 1988)(Mann et al., 1990). The rate-limiting combination of fVa and fXa, each with separate combining sites on the vesicle surface, are the initial steps required for the formation of the prothrombinase complex (Krishnaswamy, 1990) (see figure 4). The formation of the protein-phospholipid binary complexes are mutually exclusive, indicating that fXa and fVa bind to identical or overlapping sites on the membrane surface (Krishnaswamy, 1990).

Subsequent reaction(s) between membrane-bound proteins are thought to represent rapid reaction steps involving "translational" or "rotational diffusion" of the protein-phospholipid binary complexes on the vesicle surface to form prothrombinase.



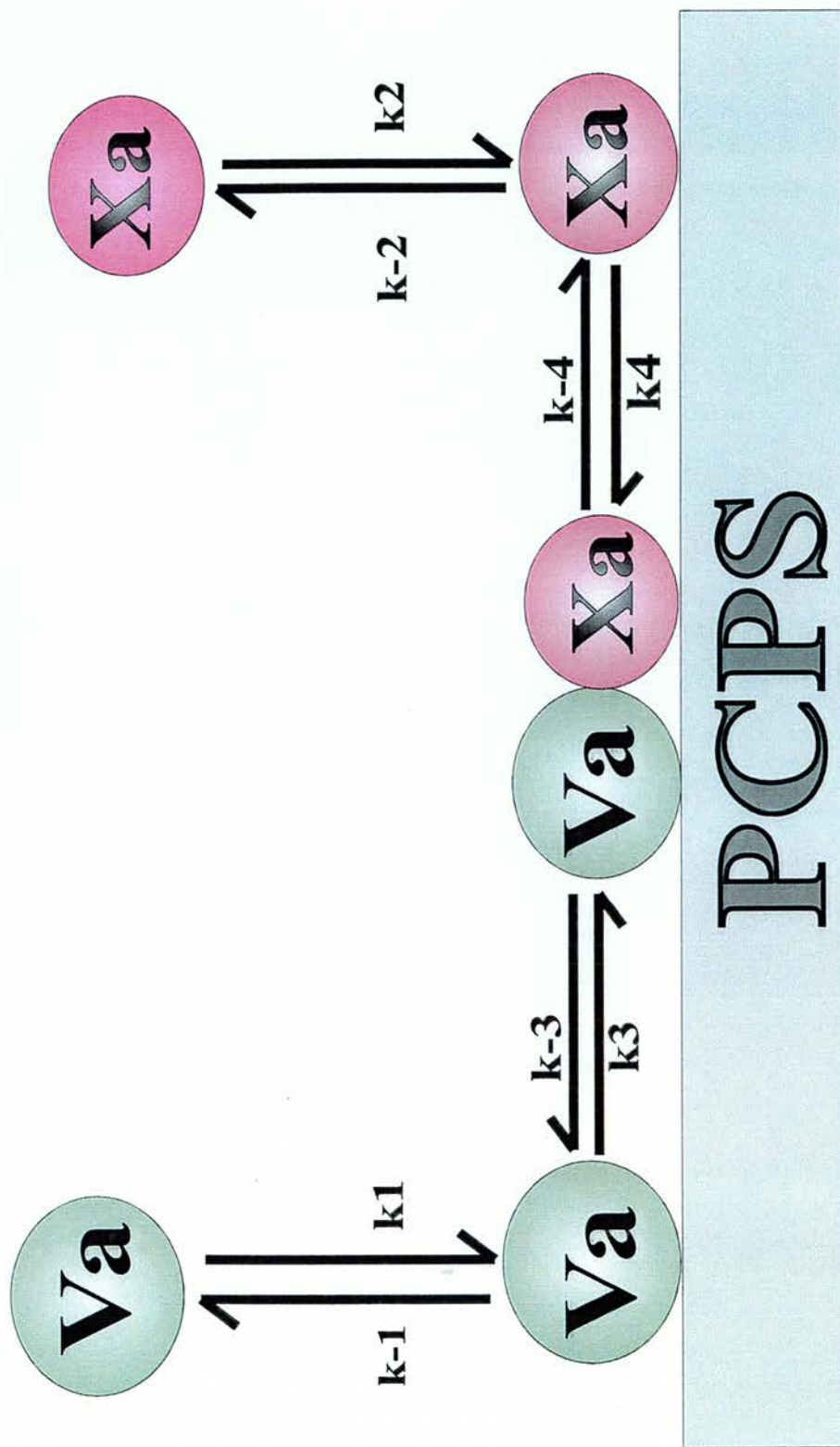


Figure 4 : Representation of the reaction steps required for prothrombinase assembly. Prothrombinase assembly on the phospholipid surface (PCPS) proceeds after initial reactions that yield separate Xa-PCPS and Va-PCPS binary complexes. The two initial association steps are governed by rate constants  $k_1$  and  $k_2$ . The PCPS-bound proteins then react rapidly on the vesicle surface (rate constants  $k_3$  and  $k_4$ ) to form prothrombinase. (Adapted from Mann, Nesheim, Church, Haley, and Krishnaswamy, 1990)

(Krishnaswamy, 1990).

### **Catalytic Advantage of Prothrombinase Complex Formation**

The catalytic advantage associated with formation of prothrombinase is shown by the data in Table I, which compare relative rates of prothrombinase activity catalysed by different combinations of components of the enzyme complex at physiological concentrations (Mann et al., 1988)(Nesheim, 1984)(Mann, 1987). The relative rate increases 300,000-fold when the complete complex is assembled (Nesheim et al., 1979)(Mann, 1987).

Kinetic studies show that the phospholipid component contributes to a reduction in  $K_m$  (the Michaelis constant, the substrate concentration at which the reaction rate is half maximal, and a measure of the affinity of the enzyme for the substrate) while fVa contributes to a rise in  $k_{cat}$  (a rate constant for the activation of the substrate at saturating concentration) (Mann, 1987)(Nesheim, 1984)(Rosing et al., 1980)(Nesheim, 1979). The mechanism responsible for each of these effects will be discussed in turn.

### **The Contribution of the Membrane Surface to the Enhanced Catalytic Rate of Prothrombinase :**

The plasma membrane consists of a complex array of lipids and protein in the form of a lipid bilayer interspersed with penetrating or attached proteins (Morrow et al., 1994). The distribution of lipids has been established experimentally by the use of phospholipases and chemical labels (Schick et al., 1976)(Bever et al., 1982a). In both erythrocyte and platelet plasma membranes, the negatively charged phospholipids, phosphatidyl serine and phosphatidylinositol, which possess potent procoagulant activity

**TABLE I****Relative Rates of Prothrombin Activation in the Presence of Various Combinations of the Components of the Prothrombinase Complex**

<u>Components present</u>	<u>Relative Rate (%)</u> *
Xa, Ca <sup>2+</sup> , phospholipid, Va	100
Xa, Ca <sup>2+</sup> , Va	0.13
Xa, Ca <sup>2+</sup> , phospholipid	0.008
Xa, Ca <sup>2+</sup>	0.0007
Xa	0.0003

Proteins are present at physiological concentration:

Prothrombin : 10<sup>-6</sup> M

fVa : 10<sup>-8</sup> M

fXa : 10<sup>-9</sup> M

Phospholipid is present at a concentration adequate to saturate the reaction

\* Relative rates are expressed in comparison to prothrombinase.

(Adapted from Mann, 1987)



(Zwaal et al., 1977) are almost exclusively located in the inner leaflet (Bevers et al., 1982b).

Platelet activation by collagen or thrombin, or by calcium ionophore, causes transbilayer movement of phospholipids resulting in increased exposure of phosphatidylserine in the outer leaflet of the membrane bilayer (Bevers et al, 1982a) (Comfurius, 1996). Although transverse diffusion of phospholipids ("flip-flop")(Bevers et al., 1983) is an extremely slow process in vesicles of pure phosphatidylcholine, as the polar head group of this molecule limits its passage through the hydrophobic lipid bilayer (Higgins, 1994), rapid "flip-flop" in artificial and natural membranes has been reported (Op Den Kamp, 1979) and is an active process requiring ATP (Devaux et al., 1991). Membrane proteins ("flippases") have recently been identified and cloned (Zhou, 1997) which catalyse rapid calcium-dependent (Zhou et al., 1998) (Ruetz and Gros, 1994) transbilayer movement of phospholipids (Hensleit et al., 1990); this "phospholipid scramblase" activity may be involved in the loss of lipid asymmetry that occurs during cellular activation, injury or apoptosis (Comfurius et al., 1996).

The mechanism by which phosphatidylserine-containing membranes enhance activity of the fIXa-VIIIa complex and fXa-fVa complex has been an area of considerable interest in the current literature (Mann et al., 1990). Originally, the membrane was believed to enhance the assembly of enzyme, cofactor and substrate (Nesheim, 1984a)(Nesheim et al., 1984b) (see appendix (i)). There is now evidence that individual phospholipid molecules alter the catalytic process for the cofactor-enzyme complex, in addition to enhancing enzyme-substrate binding (Gilbert and Arena,

1996)(Gilbert and Arena, 1997). In support of this concept, Kung and colleagues reported that membranes composed entirely of saturated phospholipids allow normal assembly of fXa and fVa to take place (Kung et al., 1994), with a normal Michaelis constant for prothrombin, but the catalytic step is greatly slowed. Additionally, Koppaka and coworkers demonstrated that soluble phosphatidylserine binds fXa and alters its catalytic activity (Koppaka et al., 1996).

### **The Contribution of Activated Cofactor to the Enhanced Catalytic Rate of Prothrombinase :**

Factor Va accelerates the catalytic rate of prothrombinase by increasing the  $k_{cat}$  for product formation. Historically, this has been attributed to an increase in the binding of the enzyme to the phospholipid surface and to alterations in the reaction pathway that occur in the presence of fVa. In the presence of fVa, the initial cleavage of (bovine) prothrombin occurs at the Arg<sup>323</sup>-Ile<sup>324</sup> bond generating meizothrombin as the principal intermediate (Rosing and Tans, 1988)(Krishnaswamy et al., 1986); in contrast, without fVa, the initial cleavage of (bovine) prothrombin occurs at Arg<sup>274</sup>-Thr<sup>275</sup>, generating prethrombin-2, and subsequently thrombin, at a much slower rate.

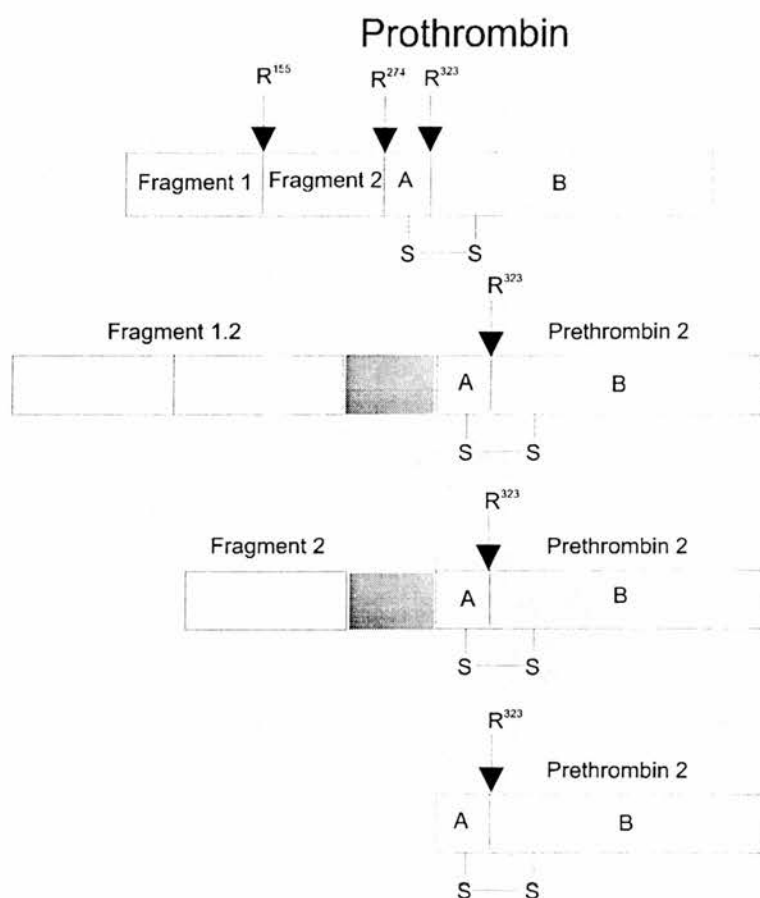
In addition to the above mechanisms, the accelerating effects of fVa appear related to the macromolecular substrate specificity of fXa (Krishnaswamy and Betz, 1997)(Betz and Krishnaswamy, 1998). Studies with tick anticoagulant peptide (TAP), a potent and highly selective fXa inhibitor obtained from the soft tick, *Ornithodoros moubata* (Waxman et al., 1990), and mutant TAP derivatives, have provided evidence for fVa-induced changes at extended macromolecular recognition sites (or "exosites")

in fXa upon its incorporation into prothrombinase (Betz et al., 1997). It is possible that the function of fVa is to enhance a number of substrate binding steps in a multistep pathway that governs the overall catalytic activity of the enzyme complex (Krishnaswamy and Betz, 1997)(Betz and Krishnaswamy, 1998).

A model has recently been proposed by Krishnaswamy and colleagues which links the ability of fVa to enhance the catalytic efficiency of fXa within prothrombinase, with the narrow macromolecular substrate specificity of fXa (Betz and Krishnaswamy, 1998). Kinetic studies of prethrombin-2 cleavage by prothrombinase have been performed in the presence or absence of prothrombin fragments 1 and 2. Prethrombin-2.fragment 1.2 is an activation intermediate formed by cleavage of prothrombin at Arg<sup>274</sup>-Thr<sup>275</sup>. Fragment 1, which contains the Gla-domain, mediates the high affinity interaction of prothrombin with the phospholipid membrane and fragment 2 is responsible for binding to fVa. Prethrombin-2 is unable to interact with either the surface membrane or fVa by itself (Suttie et al., 1977)(see figure 5). Studies have shown that occupation of the active site of the enzyme, fXa, by inhibitors or peptidyl substrates, does not alter the affinity of fXa for prethrombin-2 (Krishnaswamy and Betz, 1997). From these observations, a scheme has been proposed involving an ordered two-step binding process. The first step involves binding of substrate via its exosite, whereas the second step involves interaction at the active site prior to bond cleavage and product release (Betz and Krishnaswamy, 1998). This kinetic scheme is represented in figure 6.

### **1.2.2. The Intrinsic Tenase Complex**

Intrinsic tenase is composed of the trypsin-like serine protease, fIXa, in

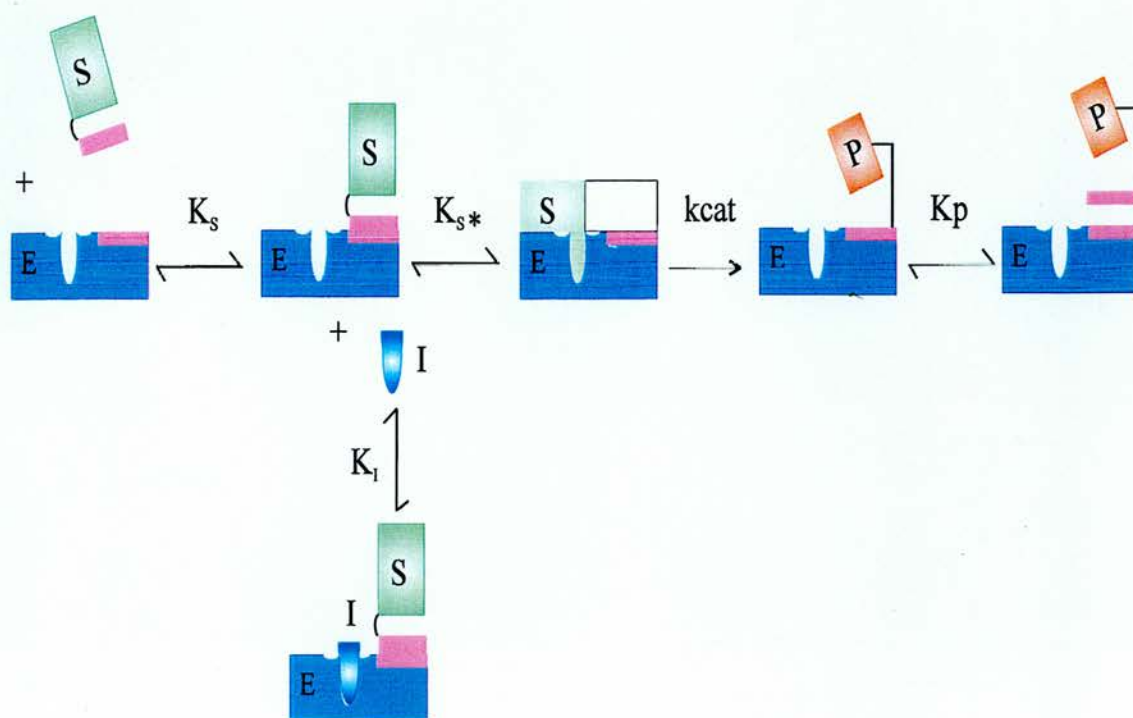


**Figure 5 : Schematic diagram of cleavage sites in prothrombin and derivatives of prethrombin-2.** (Adapted from Krishnaswamy and Walker, 1997).

Thrombin formation requires cleavage of prothrombin at Arg<sup>323</sup>-Ile<sup>324</sup> ( $R^{323}$ ) forming meizothrombin, followed by Arg<sup>274</sup>-Thr<sup>275</sup> ( $R^{274}$ ) by prothrombinase. In the absence of factor Va, cleavage at  $R^{274}$  forms prethrombin 2 plus fragment 1.2. The fragment 1 domain mediates the high affinity interaction of prothrombin with membranes; fragment 2 is responsible for binding factor Va. The site denoted by  $R^{155}$  is subject to cleavage by thrombin which releases fragment 1 from either prothrombin or fragment 1.2. The shaded area represents the ability of prethrombin-2 to interact tightly with the fragment 2 domain. Prethrombin-2 requires further cleavage at  $R^{323}$  to form thrombin; prethrombin-2 cannot interact with membranes or factor Va.

Prethrombin-2. Fragment 2 can bind factor Va.

Prethrombin 2. Fragment 1.2 can bind both factor Va and membranes.

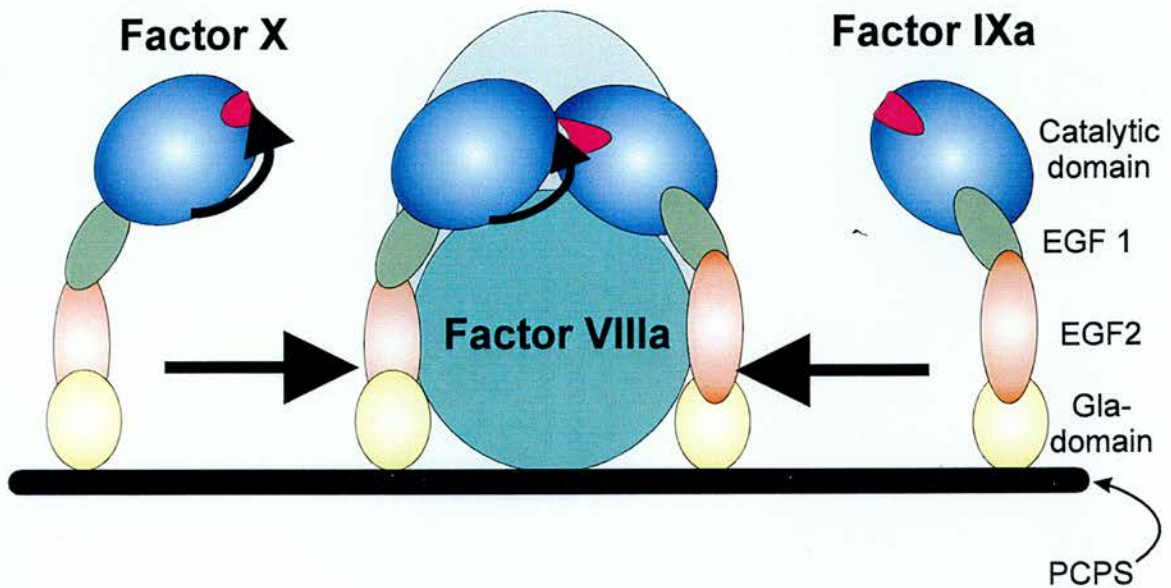


**FIGURE 6 :** Schematic diagram outlining the proposed kinetic scheme for the cleavage of substrate (prethrombin-2, S) to product (thrombin, P) by the prothrombinase enzyme complex (E) in the presence of active-site directed reversible inhibitor. (Adapted from Krishnaswamy and Betz, 1997)

association with fVIIIa on a negatively charged membrane surface, in the presence of calcium ions (Mann et al., 1988). The enzyme complex catalyses the activation of fX to fXa by means of hydrolysis of a single peptide bond at Arg<sup>194</sup>-Ile<sup>195</sup> (Lollar et al., 1993). In the presence of VIIIa, acidic phospholipid and calcium ions, the catalytic efficiency of fIXa towards fX is increased by several orders of magnitude, due to a decrease in  $K_m$  and an increase in  $k_{cat}$  for the reaction. The decrease in  $K_m$  can be accounted for by the effect of acidic phospholipids, whereas the increase in  $k_{cat}$  can be ascribed to fVIIIa (van Dieijen et al., 1981). Similar to the  $k_{cat}$  effect already described in the homologous prothrombinase complex (Rosing et al., 1980), the catalytic advantage conferred by the binding of factors VIIIa, IXa and X to the phospholipid membrane is partly attributable to the alteration of the active site structure of fIXa that occurs when it binds to fVIIIa (Mutucumarana et al., 1992).

### **Model of Intrinsic Tenase :**

The X-ray crystal structure of porcine fIXa was reported in 1995 by Brandstetter and colleagues (Brandstetter et al., 1995); fIXa resembles a "tulip" with the catalytic module representing the "flower", two interlaced EGF domains representing the "bent stalk" and the N-terminal Gla-domain representing the "bulb" (see figure 7). According to Brandstetter, the high affinity of fIXa for fVIIIa implies an extended interaction surface between the two factors, and binding sites of fVIIIa extend along the entire concave surface of the fIXa molecule (Brandstetter et al., 1995). It is proposed that the arched fIXa lies across fVIIIa, with the fIXa surface providing most of the contacts. The binding of fX to the opposite side of fVIIIa may force fXa cleavage sites close to the



**FIGURE 7 : MODEL of INTRINSIC TENASE**

Schematic diagram of a model of the intrinsic tenase complex (Brandstetter et al., 1995). The binding of fX to the opposite side of fVIIIa forces fIXa cleavage sites close to the substrate binding site of fIXa and provides the energy for "active site rigidification". (Adapted from Brandstetter et al., 1995)



substrate binding site of fIXa and provides the energy for "active site rigidification".

Factor VIII binding sites for fIXa have been localised to amino acid residues Ser<sup>558</sup>-Gln<sup>565</sup> and Arg<sup>698</sup>-Ser<sup>710</sup> of the A<sub>2</sub> domain (Fay et al., 1994) and residues 1811-1818 of the A<sub>3</sub> domain (Lenting et al., 1996).

### **Model for the Instability of the Intrinsic Tenase Complex :**

The activity of intrinsic tenase decays with time, and reflects the lability of fVIIIa (Fay et al., 1996). This was originally observed and referred to as a "self-damping" by Jesty, who observed that the rate and yield of fIXa formed by intrinsic tenase decreased with increasing fIXa concentration and a constant level of fVIIIa (Jesty, 1990).

Two mechanisms potentially contribute to this decay :

- (1) the inter-factor VIIIa subunit affinity between the A<sub>2</sub> subunit and A<sub>1</sub>/A<sub>3</sub>-C<sub>1</sub>-C<sub>2</sub> dimer (Lamphear and Fay, 1992) and,
- (2) the inactivation of fVIIIa resulting from fIXa-mediated proteolysis of the A<sub>1</sub> subunit (O'Brien et al., 1992).

Factor VIIIa is a labile heterotrimer composed of the A<sub>1</sub>, A<sub>2</sub> and A<sub>3</sub>-C<sub>1</sub>-C<sub>2</sub> subunits. The A<sub>1</sub> and A<sub>3</sub>-C<sub>1</sub>-C<sub>2</sub> subunits are linked by metal ion bridge(s) forming a structurally stable yet inactive dimer (Fay et al., 1991b). The A<sub>2</sub> subunit is weakly associated with the dimer (K<sub>d</sub>=260nM)(Lollar et al., 1992) by electrostatic interactions (Fay et al., 1991a), and dissociates at physiological pH, resulting in loss of fVIIIa activity (Fay et al., 1996). Heterotrimeric human fVIIIa activity can be efficiently reconstituted from the isolated A<sub>2</sub> subunit and A<sub>1</sub>/A<sub>3</sub>-C<sub>1</sub>-C<sub>2</sub> dimer at slightly acidic pH (Fay et al., 1991a). It has been shown that porcine fVIIIa can be stabilised by fIXa in the



presence of phospholipid and calcium; the active site of fIXa is not necessary for this effect since fIXa modified with dansyl-Glu-Gly-Arg-CH<sub>2</sub>Cl (DEGR-CK), an active site inhibitor, yields results similar to wild-type fIXa (Lollar et al., 1984). Prolonged interaction of fVIIIa with fIXa results in loss of fVIIIa activity due to proteolytic cleavage within the A<sub>1</sub> subunit and is a concentration dependent effect (Lamphear and Fay, 1992).

The relative importance of each mechanism towards the overall instability of intrinsic tenase is dependent upon underlying reactant concentrations (Fay et al., 1996). At low reactant concentrations, (for example, 0.5nM fVIIIa and 5nM fIXa), intrinsic tenase decay is determined by a weak affinity interaction between the fVIIIa A<sub>2</sub> subunit and A<sub>1</sub>/A<sub>3</sub>-C<sub>1</sub>-C<sub>2</sub> dimer, and the residual activity of the enzyme complex reaches an equilibrium. Fay and colleagues carried out an analysis using a mutant form of fVIII (fVIII<sub>R336I</sub>), which possesses an altered fIXa cleavage site. Similar rates of intrinsic tenase decay (0.12 min<sup>-1</sup>) were shown confirming the lack of contribution of proteolysis under these conditions (Fay et al., 1996). When the concentration of fIXa was increased ten-fold, the initial rate of decay of intrinsic tenase containing native fVIIIa increased (0.12 to 0.82 min<sup>-1</sup>) and paralleled the rate of proteolysis of the A<sub>1</sub> subunit (Fay et al., 1996). These, and other results, suggest a model in which decay of intrinsic tenase results from both fVIIIa subunit dissociation and fIXa-mediated cleavage, depending upon the relative concentration of reactants (Fay et al., 1996).

### 1.3. Heparin and Low Molecular Weight Heparin

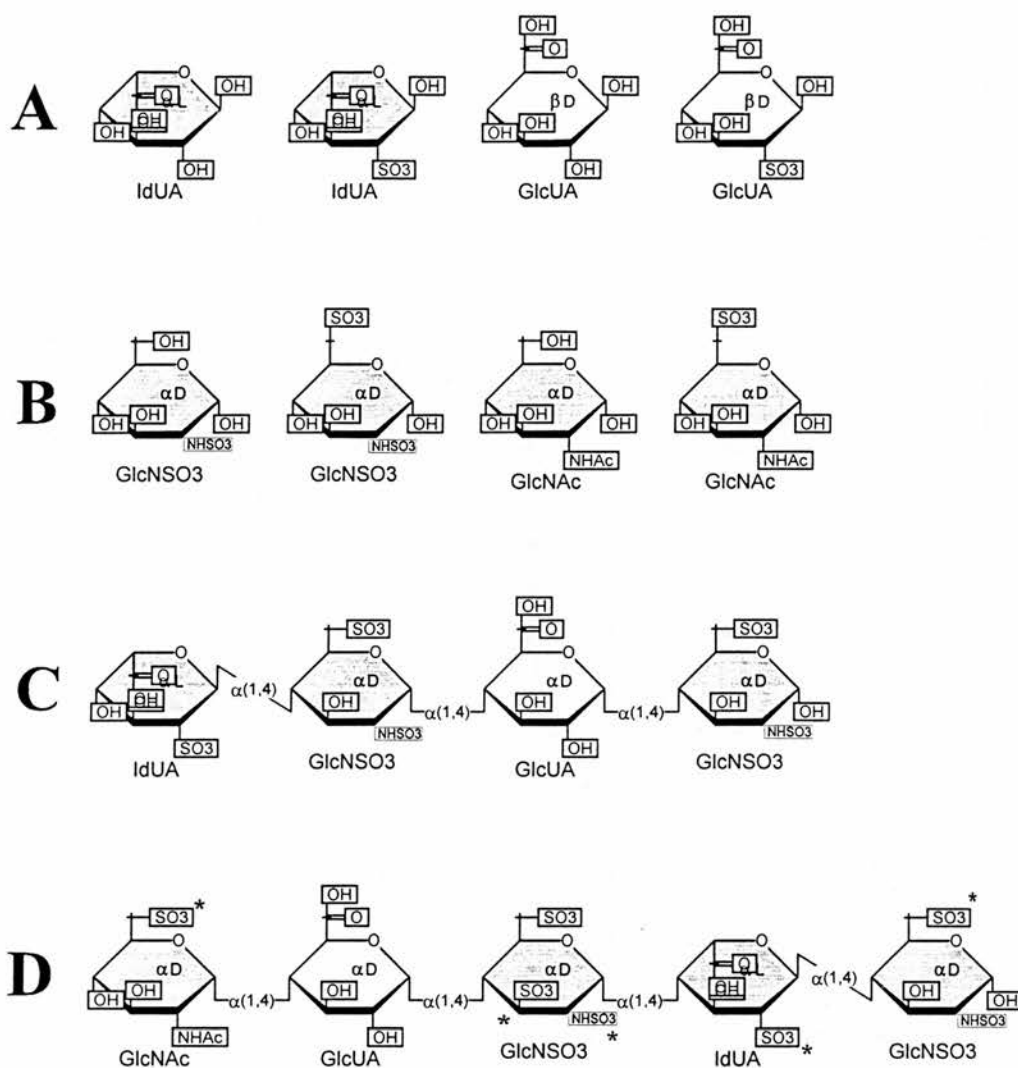
#### 1.3.1. Structure of Heparin

Heparin is a sulphated glycosaminoglycan composed of alternating residues of an uronic acid and D-glucosamine (Bjork and Lindhal, 1982)(Rosenberg and Bauer, 1994)(Hirsh et al., 1995). Uronic acid residues are either L-iduronic acid (IdUA) or D-glucuronic acid (GlcUA)(see panel 'A', figure 8), and D-glucosamine residues are either N-sulphated (GlcNSO<sub>3</sub>) or N-acetylated (GlcNAc)(panel 'B', figure 8).

Commercial preparations are usually isolated from porcine intestinal mucosa or bovine lung, and are heterogeneous mixtures of polysaccharide chains ranging in molecular weight from 3000 to 30,000, with a mean molecular weight of 15,000 (Hirsh 1991).

The amino sugar components may possess different degrees of sulphation; a small proportion of residues are 3-O-sulphated, and the 3-O-sulphated GlcNSO<sub>3</sub> residues can be either trisulphated or disulphated (Lindahl et al., 1980).

An unique combination of disaccharide units makes up the pentasaccharide sequence (panel D, figure 8), that contains the high affinity binding site for antithrombin (Lindahl et al., 1980)(Casu et al., 1981). This sequence occurs in only about one-third of heparin chains and is randomly distributed (Andersson et al., 1976)(Hirsh, 1995). Sulphate groups essential for high-affinity binding to antithrombin are shown by an asterisk in panel D, figure 8 (Thunberg et al., 1982)(Andersson et al., 1976) (Casu, 1989).



**Figure 8 :**

**Schematic diagram showing the monosaccharide units identified in heparins and typical heparin sequences.** (adapted from D.A. Lane, U. Lindhal, 1989)

**Panel A :** Uronic acid residues, either L-iduronic acid, or D-glucuronic acid, which may be variably sulphated at carbon position 2.

**Panel B :** D-glucosamine residues, which may be either N-sulphated or N-acetylated.

**Panel C :** represents a typical heparin sequence; the amino sugar components may possess different degrees of sulphation, and a small proportion of N-sulpho- $\alpha$ -D-glucosamine residues may be 3-O-sulphated; only approximately 10-20% are glucuronic acid residues.

**Panel D :** represents the unique combination of disaccharide units that makes up the "pentasaccharide sequence" which contains the high affinity binding site for antithrombin. Sulphate groups essential for high-affinity binding to antithrombin are shown by an asterisk.

### 1.3.2. Anticoagulant Effects of Heparin :

Heparin acts as an anticoagulant by activating antithrombin; the heparin-antithrombin complex then inactivates thrombin, fXa and other activated clotting factors (Björk and Lindhal)(Hirsh, 1991). Recently, studies in buffer systems have shown that heparin also exerts an antithrombin-independent anticoagulant effect by blocking the activity of intrinsic tenase (Barrow et al., 1994a). In plasma systems, however, the antithrombin-dependent effect of heparin predominates.

#### Antithrombin-dependent Anticoagulant Effect :

Antithrombin (previously known as "antithrombin III" and "heparin cofactor")(Perry, 1994) has a molecular weight of 58,200 (Olson and Björk, 1994) and a prolate ellipsoid shape. The complete primary structure was reported by Petersen and colleagues in 1979 (Petersen, 1979).

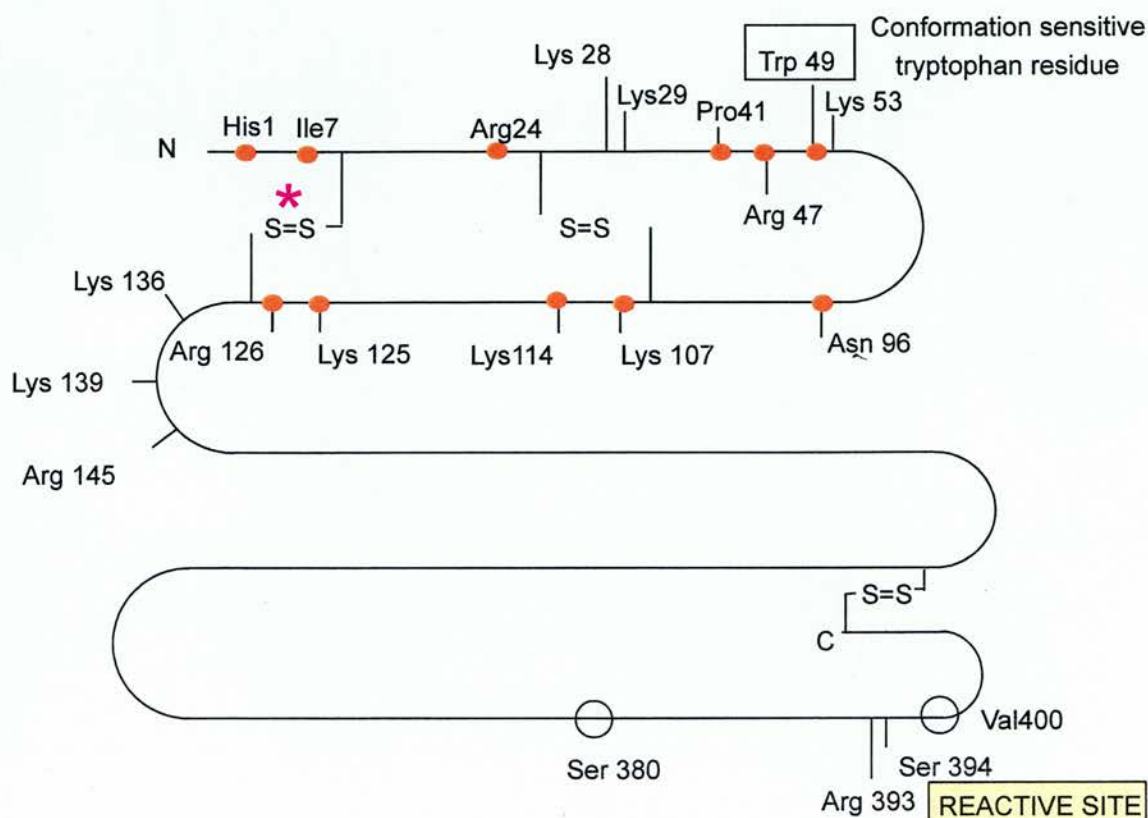
Antithrombin is a single chain glycoprotein with 432 amino acid residues (Rosenberg,1987). The mature protein has 3 disulphide bridges formed by cysteine residues Cys<sup>8</sup>-Cys<sup>128</sup>, Cys<sup>21</sup>-Cys<sup>95</sup> and Cys<sup>247</sup>-Cys<sup>430</sup> (Olson and Björk,1994)(Chandra et al., 1983)(Rosenberg, 1987), and four potential glycosylation sites at Asn<sup>96</sup>, Asn<sup>135</sup>, Asn<sup>155</sup> and Asn<sup>192</sup> (Perry, 1994)(Olson and Björk, 1994). A minor pool of normal antithrombin (10% of the total plasma concentration) known as "AT<sub>β</sub>" lacks the carbohydrate side-chain on Asn<sup>135</sup> and is not glycosylated, resulting in a slightly higher heparin affinity compared with the predominant form, "AT<sub>α</sub>" (Perry, 1994)(Lane et al., 1996).

The location of "critical sites" within antithrombin, the reactive site, the potential

heparin binding sites, the conformation-sensitive tryptophan, and the disulphide crosslinks is shown in figure 9 (Rosenberg and Bauer, 1994)(Colman et al., 1994).

In 1973, Rosenberg and Damus demonstrated that antithrombin neutralises the activity of thrombin by forming a 1:1 stoichiometric complex between the reactive site (Arg<sup>393</sup>)(also known as P1 residue), of antithrombin, and the active centre (Ser<sup>394</sup>) of thrombin (Rosenberg, 1975)(Rosenberg and Damus, 1973). Rapid kinetic studies have demonstrated that the inactivation of thrombin by antithrombin is a two step process. Initially the two reactants form a weak encounter complex, with a dissociation constant of approximately  $1.4 \times 10^{-3}$  M, which is converted to a stable complex at a rate constant of approximately  $10 \text{ s}^{-1}$  (Olson and Shore, 1982). In the absence of heparin, the apparent second order rate constant for the inactivation of thrombin is  $0.7$  to  $1.4 \times 10^{-4} \text{ M}^{-1} \text{ s}^{-1}$  at  $25$  to  $37^\circ\text{C}$ , and is increased to  $1.5$  to  $4 \times 10^{-7} \text{ M}^{-1} \text{ s}^{-1}$  in the presence of optimal concentrations of heparin, increasing the reaction rate by 2000-fold (Olson and Björk, 1994).

Heparin preparations are characterised by a subpopulation of molecules with high affinity for antithrombin (Hook et al., 1976). The high-affinity fraction, which constitutes about one third of unfractionated heparin, and contains a unique pentasaccharide unit, has high anticoagulant activity and accounts for about 90% of the activity of commercial heparin (Choay et al., 1983)(Olson and Björk, 1994). The remaining low-affinity heparin has low specific activity (<10% of the high affinity fraction). Most high affinity chains contain only one pentasaccharide sequence, randomly distributed along the chains, although some longer chains may contain two



**Figure 9 : "Critical sites" within antithrombin** : Arg-393-Ser-394 is the reactive of antithrombin. Trp-49 is the conformation-sensitive aromatic residue. The various lysine and arginine residues are potential sites for the binding of the different domains of heparin to the protease inhibitor.

(Adapted from Colman, Hirsh, Marder, Salzman, 1994)

### KEY

- carbohydrate attachment sites
- exposed loop on protein surface
- \* disulphide bridge important for heparin binding conformation

pentasaccharide units (Olson and Björk, 1994).

Four negatively charged sulphate groups in the pentasaccharide sequence bind to a single positively charged site on antithrombin ( $K_d = 1-2 \times 10^{-8} \text{ M}$ , at pH 7.4, ionic strength 0.15) (Olson and Björk, 1994)(Kridel and Knauer, 1997) located on and adjacent to the D-helix (see figure 9). Studies using stopped flow fluorimetry have highlighted the kinetics of high affinity heparin binding to human antithrombin. Binding to the pentasaccharide units results in a conformational change which is thought to take place at the reactive site loop of antithrombin, converting it into a very rapid inhibitor (Olson and Björk, 1994)(Huntington, J. A. et al., 1996).

Heparin binds to "anion-binding exosite II" on thrombin (Stubbs and Bode, 1993), an electropositive patch located on the surface of thrombin, remote from the catalytic site. The interaction of heparin with thrombin is pentasaccharide-independent and involves any number of non-specific sites on the heparin molecule. Subsequent diffusion along the surface of the polysaccharide allows thrombin to interact with antithrombin. Thus, heparin promotes a thrombin-antithrombin interaction through a "surface approximation" or "bridging" of the two proteins on the heparin surface (Olson et al., 1993). Heparin molecules require at least 13 saccharide units in addition to the pentasaccharide unit for bridging of thrombin to occur (Olson et al., 1993). Virtually all unfractionated heparin molecules that possess the pentasaccharide sequence contain at least 18 saccharide units (5400Da). In contrast, only 25-50% of low molecular weight heparin chains are of sufficient length to bind both thrombin and antithrombin. Heparin molecules that contain fewer than 18 saccharides are too short to bind both thrombin and

antithrombin simultaneously, and are unable to fully accelerate the inactivation of thrombin by antithrombin, but retain the ability to catalyse the inhibition of factor Xa by antithrombin (Olson et al., 1993)(Weitz,1997). Consequently, unfractionated heparin has equivalent activity against fXa and thrombin, and has an anti-fXa to anti-IIa ratio of 1:1, whilst low molecular weight heparins (LMWHs) (mean MW 4000-6500) have greater activity against fXa, and have anti-fXa to anti-IIa ratios of between 4:1 and 2:1(Weitz, 1997)(Hirsh and Levine, 1992).

### **Antithrombin-Independent Mechanisms of Inhibition :**

In a discontinuous plasma-free assay of fX activation by intrinsic tenase, using purified human and porcine coagulation factors, Barrow and colleagues demonstrated that both unfractionated heparin and LMWH directly inhibit the activation of fX, with  $IC_{50}$  values of 1  $\mu$ g/ml, and 6  $\mu$ g/ml respectively (Barrow et al., 1994a). In contrast, they have no effect on prothrombinase activity at concentrations up to 10  $\mu$ g/ml. Heparin produced a small increase in extrinsic tenase activity, consistent with other data in the literature (Broze, Jr. et al., 1993).

The kinetics of fX activation in the presence of heparin were further analysed to establish the mechanism by which heparin inhibits intrinsic tenase. At fixed concentrations of fIXa, fVIIIa and phospholipid, the dependence of the initial velocity of fX activation on substrate (fX) concentration follows Michaelis-Menten kinetics, and heparin caused a non-competitive pattern of inhibition (Barrow et al., 1994a).

Heparin also directly inhibits the activation of the fVIII-vWf complex by thrombin (Barrow et al., 1994b), by inhibiting heavy chain cleavages of fVIII at Arg<sup>372</sup>



and Arg<sup>740</sup>, and light chain cleavage at Arg<sup>1689</sup>. This suggests that the recognition of fVIII by thrombin involves "exosite 2". Further studies using a recombinant exosite 2 defective mutant, "thrombin RA", and hirugen, (a synthetic N-acetylated dodecapeptide), which competitively inhibits binding of macromolecules to exosite 1, were used by Esmon and Lollar to investigate the role of anion binding exosites 1 and 2 in the activation of factors V and VIII. ("Exosite 1" is a surface patch of highly positive charge density, distinct from the active site of thrombin, which binds fibrinogen, 5<sup>th</sup> and 6<sup>th</sup> EGF-like domains of thrombomodulin and the COOH-terminal domain of hirudin) (Stubbs and Bode, 1993). It was found that hirugen inhibits fV and fVIII activation by thrombin. In addition, the catalytic efficiency of the activation of thrombin, or of the first step of fV activation by thrombin RA was only 10% that of wild-type thrombin, although the overall rate of conversion to fVa was not influenced by this mutation. These results indicate that both exosites are involved in the recognition of fV and fVIII by thrombin, although there are significant differences in the nature of thrombin's interaction with these two substrates (Esmon and Lollar, 1996).

#### 1.4. Objectives

In plasma systems, the antithrombin-dependent effect of heparin predominates. To capitalise on the antithrombin-independent effect described in recent studies in buffer systems (Barrow et al, 1994a), LMWH was chemically modified by periodate oxidation followed by borohydride reduction (Young and Hirsh, 1990), a technique that selectively cleaves bonds of non-sulphated uronic acid residues. This reduces the affinity

LMWH for antithrombin by 1500-fold (from a  $K_d$  value of 25nM to 43 $\mu$ M). After demonstrating that this chemically modified LMWH derivative inhibited intrinsic tenase to the same extent as the starting material, we then explored methods of increasing the potency of LMWH. As a first step, N-desulphated LMWH was prepared using a solvolytic desulphation technique (Inoue and Nagasawa, 1976). In contrast to the starting material, N-desulphated LMWH had minimal inhibitory activity. These findings indicated that the inhibition of intrinsic tenase was antithrombin-independent, but charge-dependent. To capitalise on the charge-dependent effect, LMWH with low affinity for antithrombin was sulphated using sulphur trioxide as a sulphate donor, providing a series of increasingly hypersulphated, low affinity, low molecular weight heparins.

The first objective was to investigate the inhibitory effect of this series of LMWHs on intrinsic tenase and prothrombinase activity in buffer systems. Using kinetic studies, I explored the possibility that an increase in the extent of sulphation of LMWH, and thereby an increase in the overall negative charge of the compound, might lead to an increase in inhibitory potency.

The second objective was to determine the mechanism of inhibition by examining the influence of a hypersulphated LMWH on partially reconstituted activation complexes to demonstrate the component(s) responsible for glycosaminoglycan sensitivity.

The third objective of this study was to establish the type of inhibition by investigation of kinetic parameters, namely, the turnover number,  $k_{cat}$ , an estimation of

the catalytic rate of the enzyme complex, the Michaelis constant,  $K_m$ , a measure of the affinity of the enzyme towards the substrate, and the apparent dissociation constant,  $K_{d_{app}}$ , a measure of the affinity of the enzyme, or the cofactor, towards the rest of the assembled complex. These measurements were derived from calculations based on classical Michaelis Menten kinetics.

## **CHAPTER 2 : EXPERIMENTAL PROCEDURES**

### **2.1 Materials**

### **2.2. Methods**

#### **2.2.1. Synthesis of Phospholipid Vesicles**

#### **2.2.2. Effect of Heparins on the Activity of Prothrombinase**

#### **2.2.3. Mechanism of Inhibition of Prothrombinase by Hypersulphated LMWH**

#### **2.2.4. Effect of Heparins on the Activity of Intrinsic Tenase**

#### **2.2.5. Mechanism of Inhibition of Intrinsic Tenase by Hypersulphated LMWH**

#### **2.2.6. Effect of Hypersulphated LMWH on the Catalytic Rate ( $k_{cat}$ ), the Michaelis ( $K_m$ ) Constant, and the Dissociation Constant ( $K_{d_{app}}$ ) for Prothrombinase.**

2.2.6.a. Determination of the Catalytic Rate for Prothrombinase

2.2.6.b. Determination of the Michaelis ( $K_m$ ) Constant for Prothrombinase

2.2.6.c. Determination of the  $K_{d_{app}}$  for Prothrombinase

#### **2.2.7. Determination of the Kinetic Parameters for Intrinsic Tenase**

#### **2.2.8. Effect of Hypersulphated LMWH on Prothrombinase and Intrinsic Tenase in an Assay Using Isolated Washed Platelets as the Membrane Surface**

#### **2.2.9. Plasma-based Coagulation Assays**

### **2.3. Statistical Analysis**

## 2.0. EXPERIMENTAL PROCEDURES

### 2.1. Materials

Human factors V, Va and IXa were obtained from Haematologic Technologies Inc. (Essex Junction, VT), and factors Xa, prothrombin, factor X and  $\alpha$ -thrombin were obtained from Enzyme Research Laboratories (South Bend, IN). Monoclonal-purified human fVIII (Hemofil) was obtained from Baxter, and recombinant fVIII (Kogenate) from Bayer Inc., Etobicoke, Ontario. Albumin-free fVIII was a gift from Dr E. Saenko, Holland Laboratory, American Red Cross, Rockville, Maryland. The purity of factors V and VIII were assessed by SDS-PAGE (Laemmli, 1970), and to exclude the possibility that fIXa was contaminated with either fXa or thrombin, or fXa was contaminated with thrombin, chromogenic assays with N-methoxycarbonyl-D-norleucyl-L-arginine-4-nitranilide-acetate(Chromozym.X) and Tosyl-glycyl-prolyl-arginine-4-nitranilide-acetate (tGPR-pNA), fXa and thrombin-directed substrates, respectively, were performed.

L- $\alpha$ -phosphatidylcholine (Type III-E from egg yolk), L- $\alpha$ -phosphatidyl-L-serine (from bovine brain) and tGPR-pNA were obtained from Sigma Chemical Co., St Louis, MO. Chromozym.tPA (N-methylsulfonyl-D-phenyl-Ala-Gly-Arg-4-nitranilide-acetate), and Chromozym.X were obtained from Boehringer Mannheim, West Germany. S2444 (L-Pyroglutamyl-glycyl-L-arginine-p-nitroaniline-hydrochloride) and S2222 (N-Benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroaniline hydrochloride) were obtained from Chromogenix, Sweden. Pefachrom-IXa (Pefa 3107) was obtained from Pentapharm.Ltd, Basel, Switzerland. Polybrene (Hexadimethrine bromide) was

purchased from Aldrich Chemical Co., Oakville, Ontario. Prostaglandin E1 was purchased from Sigma, and A23187 from Molecular Probes, Eugene, Oregon.

Unfractionated Grade 1 sodium heparin (184USP units/mg) from porcine intestinal mucosa and dextran sulphate, MW 5000, were purchased from Sigma. V20-AMS was obtained from Scientific Protein Labs, Milwaukee, WI; Supersulphated low molecular weight heparin (SS-LMWH) was obtained from Agen Biomedical, Milan, Italy; Dermatan sulphate (DS) was obtained from Mediolanum Pharm., Milan, Italy, and Enoxaparin from Rhône-Poulenc-Santé, France.

All low-affinity low molecular weight heparins (LA-LMWHs) and hypersulphated low molecular weight heparins (sLMWHs) were obtained from Vascular Therapeutics, Inc., Mountain View, CA. (see Table II) A LMWH fraction with mean MW 5000 was prepared from unfractionated heparin by nitrous acid depolymerisation. It was then chemically modified to reduce its antithrombin affinity by an oxidation procedure with sodium periodate. Aldehydes formed during the oxidation step were reduced with sodium borohydride to yield primary alcohols. (Young and Hirsh, 1990). N-desulphated low molecular weight heparin (N-DS-LMWH) was prepared by a solvolytic desulphation technique, according to the method of Inoue and Nagasawa (Inoue and Nagasawa, 1976).

**TABLE II : Table showing the series of hypersulphated LMWHs used in the assays of prothrombinase and intrinsic tenase.**

Compounds	O-Sulfation Condition	Elemental Analysis	Estimated # of sulphate per disaccharide
LA-LMWH	-	C%20.48; H%4.23; S%11.04	2.42
LA-LMWH-S1	Pyridium salt, TMS 4°C, 4hrs	C%20.62; H%4.04; S%11.44	2.49
LA-LMWH-S2	Pyridium salt, TMS 4°C,20hrs	C%19.25; H%3.95; S%12.33	2.88
LA-LMWH-S3	Bu <sub>3</sub> N salt, TMS 25°C,20hrs	C%18.59; H%3.92; S%12.54	3.03
LA-LMWH-S4	Bu <sub>3</sub> N salt, Py-S, 25°C,20hrs	-	3.9
LA-LMWH-S5	Bu <sub>3</sub> N salt, TMS 60°C,20hrs	-	3.8
SS-LMWH	Unknown	C%13.27; H%3.78; N%1.42; S%15.49	5.27

The estimation of degree of sulphation per disaccharide was calculated on the basis of two assumptions :

1. LA-LMWH is composed of a regular disaccharide structure. Each disaccharide has 12 C and X S
2. All samples are pure; there is no organic or inorganic contamination. All sulphur is in sulphate form.

$$\text{Wt of C} = 12 \times 12$$

$$\text{Wt of S} = X \times 32$$

$$\begin{aligned} \text{Wt of C} / \text{Wt of S} &= \% \text{ C} / \% \text{ S} \\ &= 144 / 32X = \text{C}\% / \text{S}\% \end{aligned}$$

$$\text{so, } X = 144 \times \text{S}\% / 32 \times \text{C}\%$$

## 2.2. Methods

*2.2.1. Synthesis of Phospholipid Vesicles :* Unilamellar phosphatidylcholine-phosphatidylserine (75%/25%,w/w) vesicles (PCPS) were prepared by modification of the method of Barenholz et al. (Barenholz et al., 1977)(Bloom et al., 1979) and assayed using an inorganic phosphate assay (Ames, 1966), outlined in appendix (ii).

Bovine brain phosphatidylserine (PS) and egg phosphatidylcholine (PC) (3:1 ratio) were measured into a KIMAX glass round bottomed tube (16 x 150mm) and dried under a stream of nitrogen. The lipid was resuspended in 5.0 ml of 20mM Tris, 0.15M NaCl (pH 7.4) and sealed with parafilm under a nitrogen atmosphere. The test tube was suspended in a Fisher Bath Sonicator, Model #FS14, using a clamp, and sonicated continuously at 4°C for one hour. The crude vesicles were then centrifuged in a Beckman Ultracentrifuge with an SW-55 swinging bucket rotor, for 3 hours at room temperature (150,000 x g). Vesicles were aliquoted from the top 3mls of supernatant from the centrifugation tube, a fraction shown previously to contain vesicles of a homogeneous size (Barenholz et al., 1977). An aliquot was saved for phosphate analysis. For storage, 0.3g sucrose (10% w/v) was added to the vesicles, and following mixing, aliquots were frozen at -70°C.

*2.2.2. Effect of Heparins on the Activity of Prothrombinase:* To examine the effects of glycosaminoglycans on the activation of prothrombin by the prothrombinase complex, the rate of thrombin generation was assayed using a modification of the method of Barrow et al. (Barrow et al., 1994a). Reactions were performed in 20mM Tris-Cl, 150mM NaCl with 0.1% polyethylene glycol (TSP buffer), pH 7.4. 750µl of a stock



solution "A" was made : 645 $\mu$ l TSP buffer, 3 $\mu$ l 6mM PCPS vesicles, 6 $\mu$ l 50nM fVa, 90 $\mu$ l 20 $\mu$ M prothrombin and 6 $\mu$ l M CaCl<sub>2</sub>, giving final concentrations of 6 $\mu$ M PCPS vesicles, 0.2nM fVa, 0.6 $\mu$ M prothrombin and 2mM CaCl<sub>2</sub>, respectively. Using a subsampling approach to monitor prothrombin activation, the reaction was initiated by addition of 10 $\mu$ l 1nM fXa (final concentration 0.1nM) to 50 $\mu$ l of stock "A" and 30 $\mu$ l TSP buffer. This was performed in the presence of 10 $\mu$ l of glycosaminoglycan, at final concentrations ranging from 1-1000 $\mu$ g/ml. Two controls were run in parallel, consisting of 50 $\mu$ l stock "A" and 40 $\mu$ l TSP buffer. Individual 10 $\mu$ l aliquots were removed at 30 second intervals into a 8 x 12 flat-bottomed, 96-well microtitre plate containing 10 $\mu$ l 100mM EDTA (final concentration 10mM EDTA), pH 7.4 to quench the activation reaction. After the final aliquots were removed, the concentration of thrombin in each well was determined by chromogenic assay, using the thrombin-directed chromogenic substrate, tGPR-pNA. 100 $\mu$ l of 200 $\mu$ M tGPR-pNA (final concentration 100 $\mu$ M tGPR-pNA), containing 0.1mg/ml polybrene to neutralise the heparin, was added to each well. The final volume of reactants in each well was 210 $\mu$ l. Chromogenic substrate hydrolysis was followed by monitoring the change in optical density every 10 seconds at 22.8°C, for 5 minutes at 405nm, on a Spectra-max 340 microplate reader (Molecular Devices).

Data Analysis : Rates of chromogenic substrate cleavage were determined using the manufacturer's software, by determining the slope from the linear portion of the A<sub>405</sub> vs time plot. A standard curve of thrombin activity, with tGPR-pNA, was used to convert optical density change to thrombin concentration. The rates of activation were calculated from the slope of the graph of thrombin concentration vs time by linear regression

analysis (Quattro Pro, version 5.0, Borland International Inc. Scott's Valley, CA). Comparison with two control reactions lacking glycosaminoglycan, run in parallel, gave a rate relative to the control. The concentration of glycosaminoglycan that produced 50% inhibition of prothrombin activation rate ( $IC_{50}$ ) was calculated to allow comparison between different glycosaminoglycans. Reactions were performed in duplicate and data from 3-6 experiments averaged for each analysis.

*2.2.3. Mechanism of Inhibition of Prothrombinase by Hypersulphated LMWH :* The susceptibility of individual components of the prothrombinase complex to inhibition by sLMWH was investigated using a series of assay systems that systematically removed or substituted individual components of the enzyme complex system. The rate of prothrombinase activation in the presence of the modified LMWH was studied in the following buffered systems in the presence of the most potent hypersulphated LMWH, 'S5'.

- a) absence of factor V or factor Va
- b) absence of phospholipid
- c) a system using prothrombin, factor Xa and calcium only (ie. absence of cofactor and phospholipid)
- d) substitution of activated factor V with unactivated factor V
- e) a system monitoring the chromogenic activity of fXa

The catalytic advantage associated with the formation of the prothrombinase catalyst is well documented in the literature (Nesheim, 1984)(Mann, 1987). To maintain approximately equivalent rates of activation, concentrations of the individual reactants

were increased individually, and the final conditions used are given below :

#### CONDITIONS

complete prothrombinase fXa/ fVa/ PCPS/ II/ Ca<sup>2+</sup> : II 1.2μM, CaCl<sub>2</sub> 4mM, PCPS 12μM, fVa 0.24nM, fXa 1nM

a. system devoid of cofactor : fXa/ Ca<sup>2+</sup>/ PCPS/ II : II 1.2μM, fXa 100nM, PCPS 12μM, CaCl<sub>2</sub> 4mM, no fV/Va

b. system devoid of phospholipid : fXa/ fVa/ II / Ca<sup>2+</sup> : II 1.2μM, no PCPS, CaCl<sub>2</sub> 8mM, fVa 12nM, fXa 10nM

c. system devoid of phospholipid, cofactor and calcium : fXa/ II : II 1.2μM, fXa 100nM

d. system substituting activated cofactor by unactivated cofactor :

fXa/ fV / PCPS / II / Ca<sup>2+</sup>: II 1.2μM, CaCl<sub>2</sub> 4mM, PCPS 6μM, fV 8nM, fXa 0.2nM

e. system monitoring the chromogenic activity of fXa : fXa/ S2222 : fXa 1nM, 200μM S2222

The rate of generation of thrombin in systems (b) and (c) remained slow despite increases in reaction concentrations, and subsampling was carried out at 15 and 30 minute intervals to allow determination of the rate of thrombin generation.

#### *2.2.4. Effect of Heparins on the Activity of Intrinsic Tenase :*

The effect of sLMWH on the activity of intrinsic tenase was determined using a similar discontinuous assay to the assay of prothrombinase activity outlined in Section 2.2.2.

The rate of fXa generation was determined by modification of the method of Barrow et al., (Barrow et al., 1994a) initially using the fXa-directed chromogenic substrate, S2222, and subsequently, Chromozym.X. The reactions were initiated by addition of the substrate, fX. A stock solution "B" was made as follows : 702μl TSP, 6μl 6mM PCPS, 6μl M CaCl<sub>2</sub>, 6μl 200nM fVIII and 30μl 200nM fIXa. (Final reactant concentrations : PCPS 24μM, CaCl<sub>2</sub> 4mM, fVIIIa 0.4nM, fIXa 4nM). 70μl 100nM α-thrombin (final concentration 10nM thrombin) was added for exactly 60 seconds at room temperature. 50μl of Stock "B" was then added to 30μl TSP buffer in the presence of 10μl of glycosaminoglycan (in final concentrations ranging from 0.01μg/ml to 10μg/ml). Two

controls were run in parallel, consisting of 50µl Stock "B" and 40µl TSP. The reaction was initiated by the addition of 10µl 3µM fX (final concentration, 300nM fX). A subsampling technique was used as described in Section 2.2.2. After the final aliquots were removed, the concentration of fXa in each well was determined, initially using the fXa-directed chromogenic substrate, S2222, and subsequently using the substrate, Chromozym X. Chromogenic substrate hydrolysis was monitored as before. Experiments were repeated using a variety of glycosaminoglycans.

Data Analysis: Data was analysed as in prothrombinase assay (Section 2.2.2), and specifically required the determination of a standard curve of fXa activity with the chromogenic substrate, S2222.

#### *2.2.5. Mechanism of Inhibition of Intrinsic Tenase by Hypersulphated Heparins:*

The mechanism of inhibition of intrinsic tenase was studied using an analogous set of assays to those outlined in Section 2.2.3 for prothrombinase. Individual components of the enzyme complex system were systematically removed or substituted, and the rates of fXa generation were studied in the following buffered systems in the presence of hypersulphated LMWH :-

- a) absence of fVIIIa or fVIII.
- b) absence of PCPS vesicles.
- c) a system using fX, fIXa and calcium only (ie. absence of fVIII/VIIIa and phospholipid)
- d) chromogenic activity of fIXa with Pefachrom.IXa.
- e) substitution of activated fVIII by unactivated fVIII.

### CONDITIONS

(full intrinsic tenase): fIXa / fX / PCPS / Ca<sup>2+</sup> / fVIIIa : CaCl<sub>2</sub> 4mM, X 300nM, PCPS 24μM, IXa 4nM, VIIIa 0.4nM

a) no cofactor : fIXa / fX / PCPS / Ca<sup>2+</sup> : CaCl<sub>2</sub> 2mM, X 400nM, PCPS 19.6μM, IXa 1μM

b) no PCPS : fIXa/ fX / Ca<sup>2+</sup>/ fVIIIa : CaCl<sub>2</sub> 4mM, X 300nM, no PCPS, VIIIa 16nM (IIa 8nM), IXa 1μM

c) no PCPS, no cofactor : fIXa/ fX / Ca<sup>2+</sup> : CaCl<sub>2</sub> 8mM, X 400nM, IXa 1μM, no PCPS, no VIII or VIIIa

d) chromogenic activity of fIXa : fIXa/ ChZ.Th : fIXa 1μM

e) substitution of activated cofactor for unactivated cofactor : fIXa/ fX/ PCPS / Ca<sup>2+</sup>/ fVIII : CaCl<sub>2</sub> 8mM, fX 300nM, PCPS 16μM, fIXa 16nM, fVIII 20nM

*2.2.6. Effect of Hypersulphated LMWH on the Catalytic Rate ( $k_{cat}$ ), ( $K_m$ ), the Michaelis Constant and the Apparent Dissociation Constant ( $K_{d_{app}}$ ) for Prothrombinase :* To further determine the type of inhibition caused by the hypersulphated LMWHs, the catalytic rate constant of the enzyme complex (turnover number,  $k_{cat}$ ), the Michaelis constant,  $K_m$ , and the  $K_{d_{app}}$  were derived kinetically.

*2.2.6.a Determination of the Catalytic Rate for Prothrombinase in the Absence and Presence of Hypersulphated LMWH:* The catalytic rate of prothrombinase was determined by monitoring the rate of thrombin generation in a continuous assay, using fXa to initiate the reaction.

9μl 10μM fVa, 135μl 80μM prothrombin and 36μl M CaCl<sub>2</sub> were mixed in the presence of 18μl 6mM PCPS vesicles in TSP buffer to make a stock solution "C". (Final reactant conditions : II 2.4μM, fVa 20nM, PCPS 24μM, CaCl<sub>2</sub> 8mM, and S2444 400μM) 250μl of stock "C" was added to 200μl TSP buffer in a 1 x 1cm cuvette, with the addition of 500μl of S2444. A magnetic stir bar was used throughout the procedure. Whilst reactants were being assembled, a baseline reading was carried out for 30 seconds. The reaction was initiated following the addition of 50μl 10nM fXa, (final concentration 0.05nM) giving a final volume of 1ml in the reaction chamber. The rate of thrombin generation was determined by monitoring the change in optical density at 405nm for 15 minutes on

a Beckman DU 7400 spectrophotometer. A standard curve of thrombin activity with S2444 was used to convert optical density change to thrombin concentration. S2444 was the chromogenic substrate of choice for this assay, as its reduced efficiency with thrombin allowed monitoring of thrombin at high rates of activation. In addition, the reactivity of this chromogenic substrate with thrombin was not inhibited by heparin.

A series of assays were carried out spectrophotometrically to determine the turnover number under different experimental conditions. Variables examined included changes in final reactant concentrations, the type of chromogenic substrate, the temperature of the reaction and pre-soaking of the reaction chamber with 1% Tween for 24 hours prior to use. Changes in these variables cause variation in the turnover numbers quoted for prothrombinase in the literature (Krishnaswamy, 1990). The effect on the catalytic rate of prothrombinase was noted and optimal conditions were chosen for subsequent determination of kinetic parameters.

The assay was adapted for use on a Spectra-max 340 microplate reader by using a smaller volume of reactants. 125 $\mu$ l of stock solution "D" was made consisting of 1 $\mu$ l 10 $\mu$ M fVa, 15 $\mu$ l 80 $\mu$ M prothrombin, 4 $\mu$ l M CaCl<sub>2</sub>, 2 $\mu$ l 6mM PCPS, and 103 $\mu$ l TSP. (These conditions gave the same final reaction conditions of prothrombin 2.4 $\mu$ M, fVa 20nM, PCPS 24 $\mu$ M and CaCl<sub>2</sub> 8mM). 50 $\mu$ l of stock "D" was added to 30 $\mu$ l TSP buffer and 10 $\mu$ l glycosaminoglycan, or to 40 $\mu$ l TSP for the control reading. 90 $\mu$ l 4mM S2444 (final concentration 400 $\mu$ M S2444) was added, and the reaction was initiated using 10 $\mu$ l 0.5nM fXa (final concentration 0.05nM fXa) giving a final volume of 200 $\mu$ l in the reaction well. Readings were taken every 11 seconds for 15 minutes, at 405nm,

at room temperature. The turnover number was determined in the absence and presence of an increasing range of hypersulphated LMWH (final concentrations of 0-1000  $\mu\text{g/ml}$ ).

Data Analysis : For both techniques,  $A_{405}$  values were imported into a spreadsheet (Quattro.Pro (version 7.0) Borland Int. Inc., Scotts Valley, CA), point to point slopes determined, and by reference to the standard curve generated for thrombin activity vs [S2444], converted to thrombin concentration, and then plotted vs time to obtain the activation rate. Data was then imported into Table Curve, Jandel. version 4.0, (San Rafael, CA), and a curve fitted to the graph of  $k_{\text{cat}}$  (nMIIa/sec/nMXa) vs concentration of GAG ( $\mu\text{g/ml}$ ), allowing determination of the  $\text{IC}_{50}$  value.

#### *2.2.6.b. Determination of the Michaelis Constant ( $K_m$ ) for Prothrombinase:*

The  $K_m$ , a measure of the relative affinity of the fVa/fXa complex for the substrate, prothrombin, was determined by measuring thrombin generation in a series of assays with fixed concentrations of enzyme and cofactor, and increasing substrate concentrations.

Simultaneous assays were performed in the absence and presence of sLMWH as follows:-

A series of nine stock solutions were made, each of final volume 125  $\mu\text{l}$ , consisting of fixed concentrations of fVa, PCPS and  $\text{CaCl}_2$ , and increasing concentrations of prothrombin (Final concentrations : 20nM Va, 24  $\mu\text{M}$  PCPS, 8mM  $\text{CaCl}_2$ , prothrombin ranging from 0 - 3.75  $\mu\text{M}$ ).

Each stock contained 1  $\mu\text{l}$  10  $\mu\text{M}$  fVa, 2  $\mu\text{l}$  6mM PCPS and 4  $\mu\text{l}$  M  $\text{CaCl}_2$ , and 15  $\mu\text{l}$  of 3  $\mu\text{M}$  - 125  $\mu\text{M}$  prothrombin. 50  $\mu\text{l}$  of stock was mixed with 40  $\mu\text{l}$  Tris-PEG buffer in a

series of wells and 90µl 4mM S2444 added (final concentration 400µM S2444). 20µl 0.5nM fXa (final concentration 0.05nM) was added to each well using a multichannel pipette to initiate the reaction, giving a final volume of 200µl. Changes in optical density at 405nm were monitored for 15 minutes on a Spectra-max 340 micro-plate reader at room temperature, and converted to the thrombin generation rate. Assays were carried out simultaneously in the presence of 10µl 10mg/ml sLMWH, giving a final concentration of 500µg/ml. Assays were performed in duplicate.

Data Analysis:  $K_m$  and  $V_{max}$  were determined by importing data into Table Curve, and fitting the data for the thrombin generation rate vs prothrombin concentration ( [II] ) to the equation (Chang et al., 1997) :-

$$V = \frac{[II] \times V_{max}}{K_m + [II]} \quad \text{and} \quad k_{cat} = \frac{V_{max}}{[limiting \text{ reactant}]}$$

*2.2.6.c. Determination of  $K_{d_{app}}$  for Prothrombinase, by Increasing the Cofactor Concentration, in the Absence and Presence of sLMWH:* The apparent  $K_d$  of fVa for prothrombinase, at saturating concentration of PCPS vesicles, was determined by measuring the rate of thrombin generation a series of assays with fixed substrate and enzyme concentrations, and increasing concentrations of cofactor.

A series of eleven stock solutions were made. Each stock had a final volume of 125µl and contained 15µl 80µM prothrombin, 4µl M  $CaCl_2$  and 2µl 6mM PCPS vesicles (final concentrations of prothrombin 2.4µM,  $CaCl_2$  8mM, and PCPS 24µM), with 1-4µl 1µM



fVa (final concentration 2-8nM fVa), and 1-5 $\mu$ l 10 $\mu$ M Va (final concentration 10-100nM fVa). A control experiment contained no fVa.

50 $\mu$ l of each stock was mixed with 40 $\mu$ l Tris-PEG buffer in a series of wells. 90 $\mu$ l 400 $\mu$ M S2444 (final concentration of 400 $\mu$ M S2444) was added, followed by 20 $\mu$ l 0.5nM fXa (final concentration 0.05nM) using a multichannel pipette, to initiate the reaction, giving a final volume of 200 $\mu$ l. The thrombin generation rate was determined as in Section 2.2.6. Assays were carried out simultaneously in the presence of 10mg/ml sLMWH, giving a final concentration of 500 $\mu$ g/ml, and were performed in duplicate. In an analogous assay, the  $K_{d_{app}}$  of fXa for prothrombinase was determined by measuring the turnover rates by varying the concentrations of fXa from 0-25nM.

Data Analysis : Data was fitted to a second order polynomial equation  $a_0 + a_1 \cdot t + a_2 \cdot t^2$ .

The  $a_2$  coefficient gives the rate of change in  $A_{405}$  and is equal to

$$k \cdot S2444 \cdot k_{II} \cdot [Xa/Va]/2$$

where  $k \cdot S2444$  is the rate that fXa cleaves S2444. The thrombin generation rate was plotted against concentration of fVa, and the affinity of fVa for fXa determined by fitting the data to the equation (Krishnaswamy, S., 1990):-

$$Xa \cdot Va = b = [(Va_1 t + Xa_1 t + kd) \pm \sqrt{(Va_1 t + Xa_1 t + kd)^2 - 4Xa_1 t + Va_1 t}]/2$$

$$rate = k \cdot b$$

### 2.2.7 Determination of the Kinetic Parameters for Intrinsic Tenase:

In an analogous series of assays, the catalytic rate ( $k_{cat}$ ), the Michaelis ( $K_m$ )

constant, and the apparent dissociation constants ( $K_d$ ) for enzyme, were determined for intrinsic tenase, in the absence and presence of sLMWH. The final reactant concentrations used for determination of the turnover number were :

fX:192nM   fVIIIa 0.48nM   fIXa 2.5nM   PCPS 40 $\mu$ M   fIXa 2.5nM

ChZX 500 $\mu$ M

Full methodology is given in appendix (iii). The critical differences in methodology from the prothrombinase assays involved are:

- (i) the prior activation of fVIII by thrombin, yielding a fVIII/VIIIa/thrombin mixture to either initiate, or participate in the intrinsic tenase reaction.
- (ii) the initiation of the reaction with the substrate, fX.

*2.2.8. Effect of Hypersulphated LMWH on Prothrombinase and Intrinsic Tenase, using Isolated Washed Platelets as the Membrane Surface.* Synthetic PCPS vesicles (75:25) do not fully mimic the platelet surface (London and Walsh, 1996)(Bever et al., 1982)(Rawala-Sheikh et al., 1990). To determine whether hypersulphated LMWHs exert different inhibitory effects in the presence of a platelet surface in place of PCPS vesicles, a series of continuous assays of prothrombinase and intrinsic tenase were carried out using an isolated washed human platelet preparation, in the presence and absence of sLMWH.

Isolation and Activation of Platelets: Platelets were isolated by modification of the procedure of Mustard et al. (Mustard et al., 1972)(Nesheim et al., 1988). Human blood was drawn from healthy volunteers and added to 3.2% sodium citrate (10:1, v/v), using

a 0.6mm gauge needle, and mixed gently without agitation. Platelet-rich plasma (PRP) was prepared by centrifugation at 190 x g for 15 minutes at 37°C. PRP was then removed and the platelets were pelleted by centrifugation at 2500 x g for 15 minutes at 37°C. The supernatant plasma was removed, and the platelets were resuspended in Tyrode's modified buffer (TMB) + Na Citrate (10ml) + PGE<sub>1</sub> (5µM), and incubated at 37°C for 15 minutes. Tyrode's buffer was prepared as described in the procedure by Yuan et al.(Yuan et al.,1996). The suspension was recentrifuged at 1200 x g for 10 minutes, resuspended in a solution of the same composition, and incubated for 10 minutes at 37°C. The procedure was repeated, but following the next centrifugation, the pellet was resuspended in TMB 2ml (no Na citrate) and kept at 37°C until use. The number of cells present were counted using a haemocytometer slide, as shown in appendix (v). The washed platelets were retained at 37°C and used within 2 hours of isolation. Immediately prior to use, platelets were activated using 10µM A23187. All steps were performed in plastic containers, and all operations involved in isolating and washing the platelets were carried out at 37°C. Assays of prothrombinase and intrinsic tenase were carried out under the following conditions, using the methodology outlined in sections 2.2.2 and 2.2.4.

**a) prothrombinase**

fVa 20nM

prothrombin 2.4µM

Ca<sup>++</sup> 8mM

fXa 0.05nM

S2444 400µM

platelets 10µl of suspension containing  $1.7 \times 10^9$  cells

**b) intrinsic tenase**

fVIII/fVIIIa/thrombin 0.48nM

fX 192nM

Ca<sup>++</sup> 8mM

ChZ.X 500μM

fIXa 2.5nM

platelets 10μl of suspension containing  $1.7 \times 10^9$  cells*2.2.12. Plasma-based Coagulation Assays*

Activated Partial Thromboplastin Time : The Activated Partial Thromboplastin Time (APTT) is a screening test for the intrinsic and common coagulation pathways, and tests factors II, V, VIII, IX, XI, XII, prekallikrein (PK) and high molecular weight kallikrein (HMWK). (Proctor and Rapaport, 1961) It acts as a suitable measure of the activities of the intrinsic tenase and prothrombinase complexes in plasma assays. APTTs were carried out using the ST4 coagulometer, and the APTT reagent used was Thrombosil (Ortho Diagnostics, NJ.), a commercial reagent containing rabbit brain phospholipid extract with a micronised silica activator. 50μl of sample (normal plasma pool, or plasma immunodepleted of antithrombin) (Affinity Biological Inc., Ontario) was added to 50μl Thrombosil and incubated for 5 minutes. 50μl CaCl<sub>2</sub> was added, and the machine timer was started. Results were recorded in seconds.

Factor Xa-Clotting Time : The fXa-clotting time is a screening test that measures the activation of prothrombin to thrombin and is sensitive to the levels of fV and prothrombin. It was used as a measure of prothrombinase activity. The procedure was carried out on an ACL 3000 centrifugal coagulation analyser. 0.02M CaCl<sub>2</sub> was added to fXa/phospholipid reagent (0.1% BSA/Cephalin solution, 1μl 1.58mg/ml fXa), and the reagent placed in a well containing normal plasma, or plasma immunodepleted of

antithrombin. The time for clot formation was monitored using the PT mode of the coagulation analyser.

Note : The experiments outlined in Section 2.2.12 were performed by M. Johnson, A.R.T., Hamilton Civic Hospitals Research Centre, Hamilton, Ontario.

### **2.3. Statistical Analysis**

All experimental values for  $IC_{50}$  reported in sections 2.2.2-5 and 2.2.8 represent the mean of 3-6 experiments performed in duplicate.

Mean and standard deviation were calculated using Quattro Pro. version 5.0 (Borland International, Inc., Scotts Valley, CA).

The correlation between mean  $IC_{50}$  values for prothrombinase and intrinsic tenase, and the degree of sulphation of LMWH was determined by using a test of rank correlation (Pearson) and also by one-way analysis of variance (ANOVA). Statistics were derived using Minitab, 1996. A value of  $p < 0.05$  was considered statistically significant.

Experimental values for kinetic parameters in Sections 2.2.6 and 2.2.7 were carried out in duplicate, and are given in full with mean  $K_m$  and  $K_{d_{app}}$  values, and their associated standard deviation.

## **CHAPTER 3 : RESULTS**

- 3.1. Effect of Hypersulphated LMWH on the Activity of Intrinsic Tenase and Prothrombinase**  
Inhibition of Intrinsic Tenase Activity by Hypersulphated LMWH  
Inhibition of Prothrombinase Activity by Hypersulphated LMWH
- 3.2 Effect of Hypersulphated LMWH on Plasma-based Coagulation Assays**
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## CHAPTER 3 : RESULTS

### 3.1. Effect of Hypersulphated LMWH on the Activity of Intrinsic Tenase and Prothrombinase :

*Inhibition of Intrinsic Tenase Activity by sLMWH* : To investigate the structural requirements for the inhibition of LMWH, LA-LMWH was first N-desulphated by a solvolytic desulphation technique (Inoue and Nagasawa, 1976). In an assay of intrinsic tenase activity, at fixed concentrations of fIXa, fVIIIa, fX and PCPS vesicles, LA-LMWH produced 50% inhibition of the initial velocity of fX activation at 16.3 $\mu$ g/ml. A LMWH with normal antithrombin affinity inhibited intrinsic tenase to the same extent, with an IC<sub>50</sub> value of 13.2 $\mu$ g/ml (see Table III). In contrast, N-desulphated LMWH had less inhibitory effect, with a 10-fold increase in the IC<sub>50</sub> value to 166 $\mu$ g/ml. These findings indicate that the inhibition of intrinsic tenase by LMWH is antithrombin-independent, but charge-dependent.

To further investigate the charge-dependent effect, LA-LMWH was progressively hypersulphated using sulphur trioxide as a sulphate donor. Table II ( see "Experimental Methods") shows the estimated degree of sulphation for each LMWH. In contrast to LA-LMWH, the most highly sulphated LMWH, "S5", was a 32-fold more potent inhibitor of intrinsic tenase with an IC<sub>50</sub> value of 0.47 $\mu$ g/ml. These results are shown graphically in figure 10. In conclusion, progressive hypersulphation of LMWH increases the inhibitory potency of the glycosaminoglycan, reflected by a progressive reduction in IC<sub>50</sub> values. These results, when subjected to regression analysis, gave a Pearson correlation coefficient of -0.86, which on one-way analysis of variance (ANOVA) gave a p value



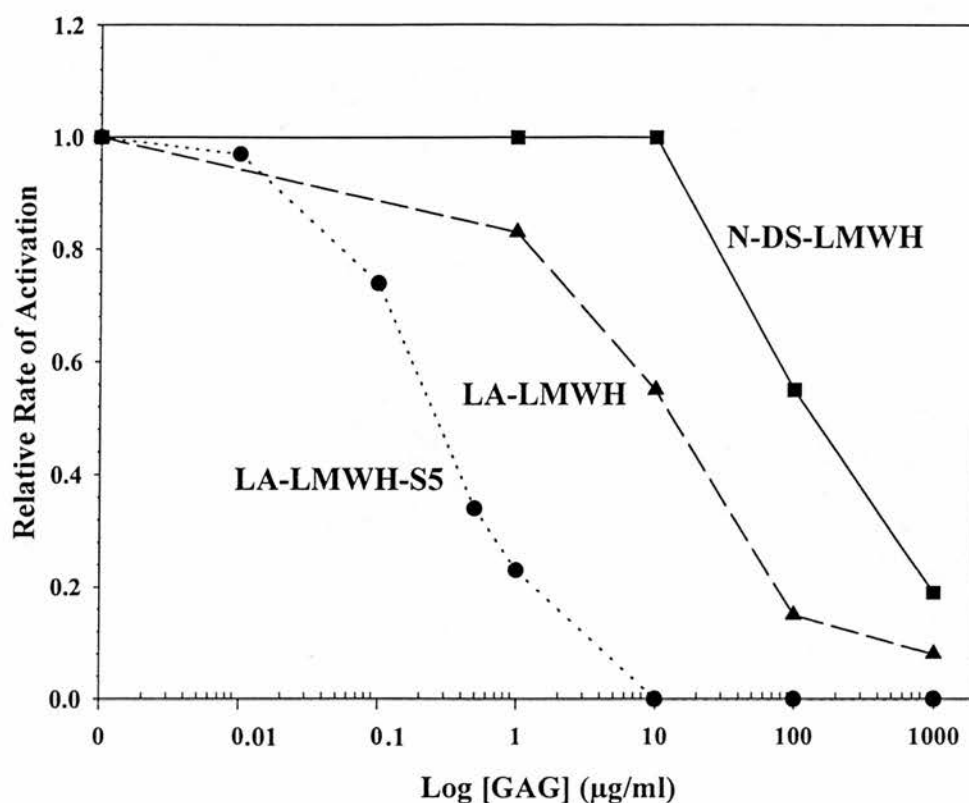


Figure 10 : Inhibition of Intrinsic Tenase by sLMWH.

Glycosaminoglycans were added at the given concentrations and the initial velocity of the activation of 300nM fX by intrinsic Xase (4nM fIXa, 400pM fVIIIa, 24μM PCPS) was determined at room temperature. The ordinate is expressed as the rate relative to the mean of 2 control readings carried out in the absence of glycosaminoglycan. Under similar reactant conditions, a turnover number of 17.9nM Xa/s/nM VIIIa was obtained.



$< 0.001$ , indicating that the potency of LMWH depends highly on its degree of sulphation. Mean  $IC_{50}$  results are given in Table III.

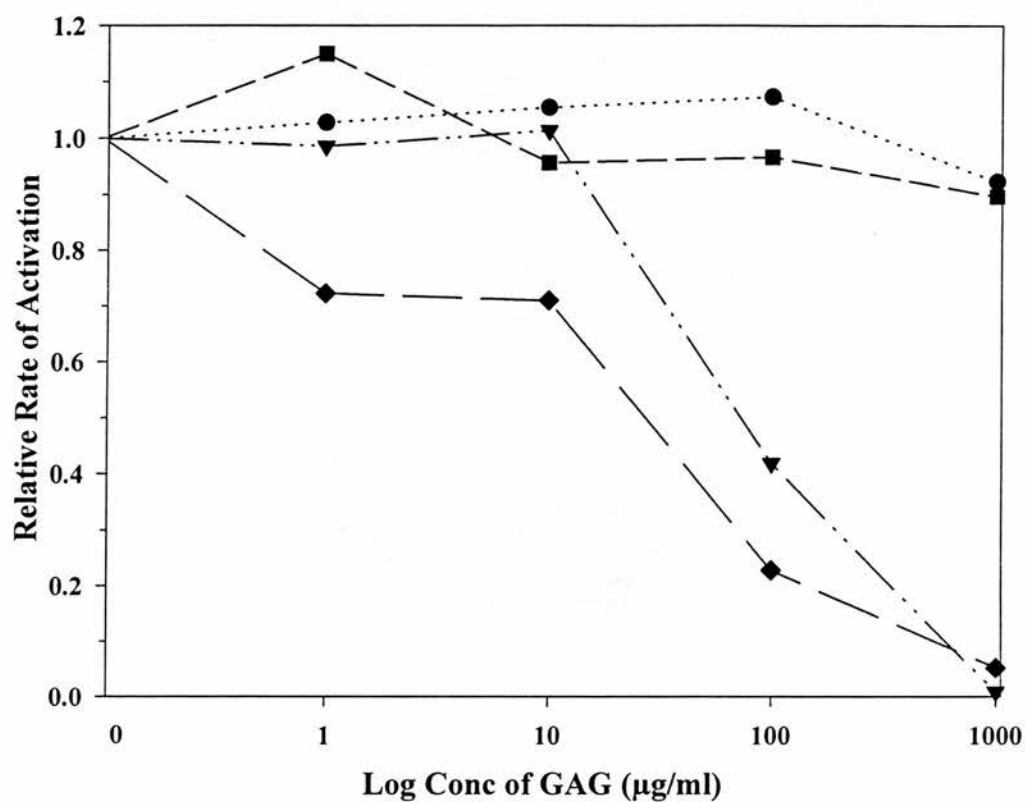
*Inhibition of Prothrombinase Activity by sLMWH :* The inhibitory effect of our series of hypersulphated LMWHs was examined on prothrombinase activity to determine if a charge-dependent inhibitory effect similar to that seen with intrinsic tenase was present. The results of these assays were expressed as mean  $IC_{50}$  values and are summarised in Table III.

Unfractionated heparin had no detectable effect on prothrombinase function at concentrations up to  $1000\mu\text{g/ml}$  (see figure 11), consistent with the results of Barrow et al. (Barrow et al., 1994a). Likewise, no inhibitory effect was demonstrated for either LA-LMWH or Enoxaparin. In contrast, hypersulphated LMWH is a potent inhibitor of prothrombinase. Similar to the inhibitory effects demonstrated in intrinsic tenase assays, by increasing the degree of sulphation of LMWH, there is a progressively greater inhibition of prothrombinase, as reflected by a steady reduction in the  $IC_{50}$  value (see figure 12).

Linear regression analysis gave a Pearson correlation coefficient of  $-0.92$ , and one-way ANOVA gave a  $p$  value  $< 0.001$ , indicating a very highly statistically significant correlation between the degree of sulphation and the mean  $IC_{50}$  values.

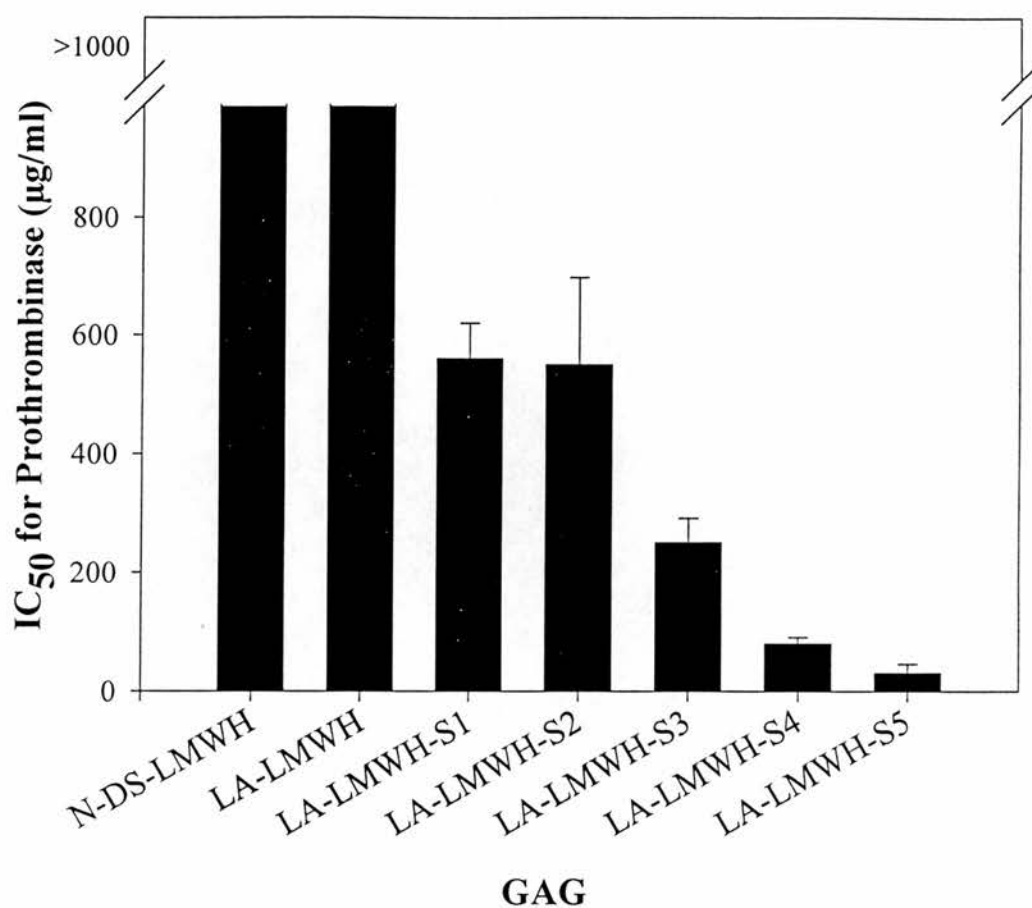
**Table III : Effect of a Series of Hypersulphated Low Affinity Low Molecular Weight Heparins on Intrinsic Tenase and Prothrombinase :** This table shows the series of LMWHs used in this study. LA-LMWH was N-desulphated by a solvolytic technique. LA-LMWH was also hypersulphated by a technique using sulphur trioxide as a sulphate donor. Results from assays of prothrombinase and intrinsic tenase activity show that as the degree of sulphation increases, the inhibitory potency of LA-LMWH increases, reflected by progressive reduction in  $IC_{50}$  values.

Compound	Estimated # of sulphation per disaccharide	Mean $IC_{50}$ Prothrombinase ( $\mu\text{g/ml}$ )	Mean $IC_{50}$ Intrinsic Tenase ( $\mu\text{g/ml}$ )
N-desulphated LMWH	1.7	>1000	$166 \pm 25$
LMWH (Enoxaparin)	2.3	>1000	$13.2 \pm 7.7$
LA- LMWH	2.42	>1000	$16.3 \pm 6.1$
LA- LMWH S1	2.49	$560 \pm 22.4$	$7 \pm 3$
LA- LMWH S2	2.88	$550 \pm 148$	$4 \pm 1.4$
LA- LMWH S3	3.03	$250 \pm 40.8$	$4.25 \pm 1.97$
LA- LMWH S4	3.82	$79 \pm 11.6$	$0.25 \pm 0.09$
LA- LMWH S5	3.9	$30 \pm 16.3$	$0.47 \pm 0.2$



**Figure 11 : Inhibition of Prothrombinase by sLMWH.**

The ordinate axis shows the rate relative to the mean of 2 controls. Unfractionated heparin (●), low affinity LMWH (LA-LMWH) (■), and 2 hypersulphated LMWHs, S4 (▼), and S5 (◆) were added at the indicated concentrations, and the initial velocity of the activation of 1.2μM human prothrombin by prothrombinase (1nM fXa, 0.24nM fVa, 24μM PCPS, 4mM  $\text{Ca}^{++}$ ) was determined at room temperature.



**Figure 12 : Effect of Increasing the Degree of Sulphation of LMWH on IC<sub>50</sub> for Prothrombinase**

The ordinate axis shows the concentration of glycosaminoglycan which causes 50% inhibition of the initial velocity of prothrombin activation (IC<sub>50</sub>, µg/ml).

Neither N-desulphated LMWH, nor LA-LMWH have an inhibitory effect on prothrombinase.

The  $IC_{50}$  values for the LMWH series were 2 orders of magnitude higher than those for intrinsic tenase, indicating that the most potent inhibitory effect of these hypersulphated LMWHs is on the intrinsic tenase complex.

In conclusion, progressive hypersulphation of LMWH increases the degree of inhibition of both enzyme complexes, and the inhibition of the intrinsic tenase and prothrombinase complexes by LMWH in buffer systems is charge-dependent.

### **3.2. Effect of Hypersulphated LMWH on Plasma-Based Coagulation Assays :**

Coagulation assays were performed to demonstrate whether the results observed in buffer systems occur in plasma systems. The activated partial thromboplastin time (APTT) was used as a measure of both intrinsic tenase activity and prothrombinase activity, and the fXa-clotting time was used as a measure of prothrombinase activity.

Whilst unfractionated heparin at therapeutic concentrations had no effect on the APTT and fXa-clotting time in plasma immunodepleted of antithrombin, low affinity, hypersulphated LMWH prolonged the APTT and fXa clotting time in AT-depleted plasma to the same extent as in control plasma (see figures 13 and 14). These results demonstrate that sulphation of LMWH effects inhibitory properties in prothrombinase and intrinsic in plasma systems, and confirms that the mechanism of inhibition is antithrombin-independent.

### **3.3. Mechanism of Inhibition of Prothrombinase and Intrinsic Tenase by sLMWH:**

As prothrombinase and intrinsic tenase are complex multicomponent systems, it

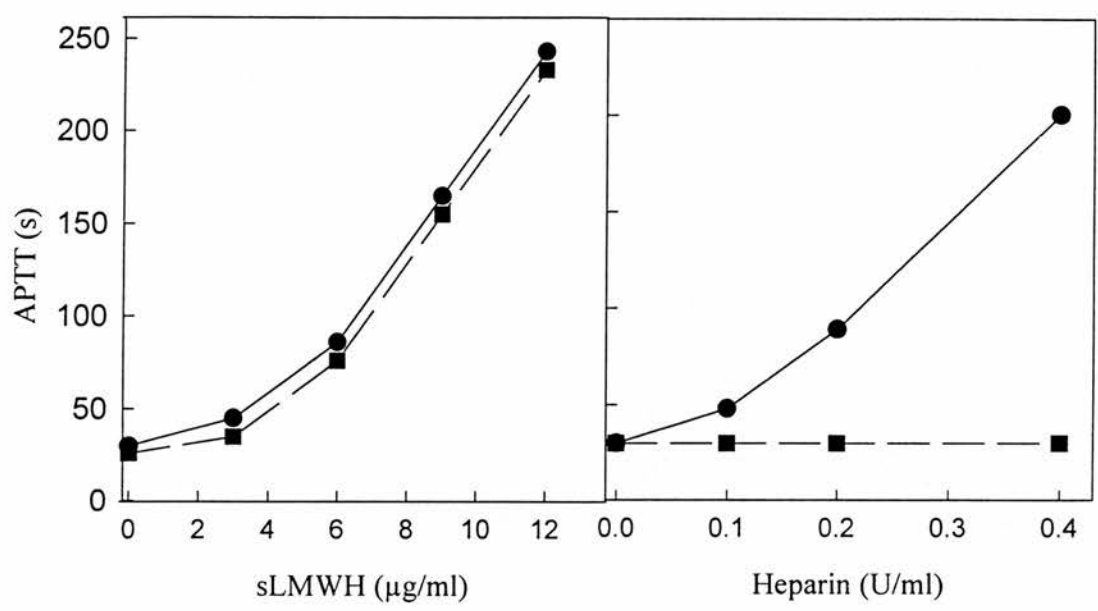


Figure 13 : Effect of hypersulphated LMWH (sLMWH) or heparin on the activated partial thromboplastin time (APTT) in control plasma (● ) or plasma immunodepleted of antithrombin. (■ ) 0.1 U/ml is equivalent to 0.5µg/ml heparin.)

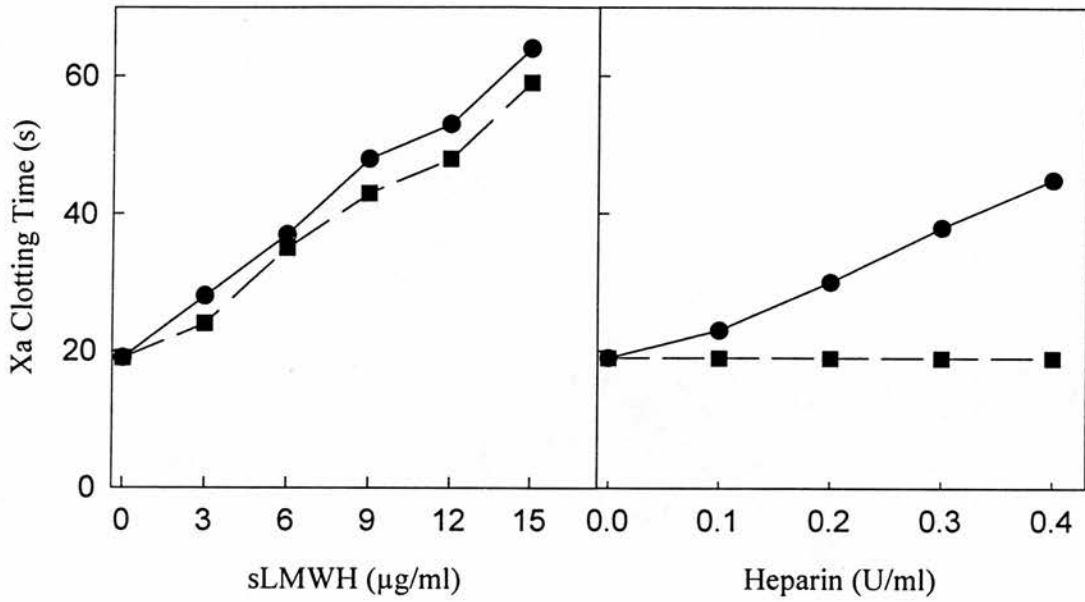


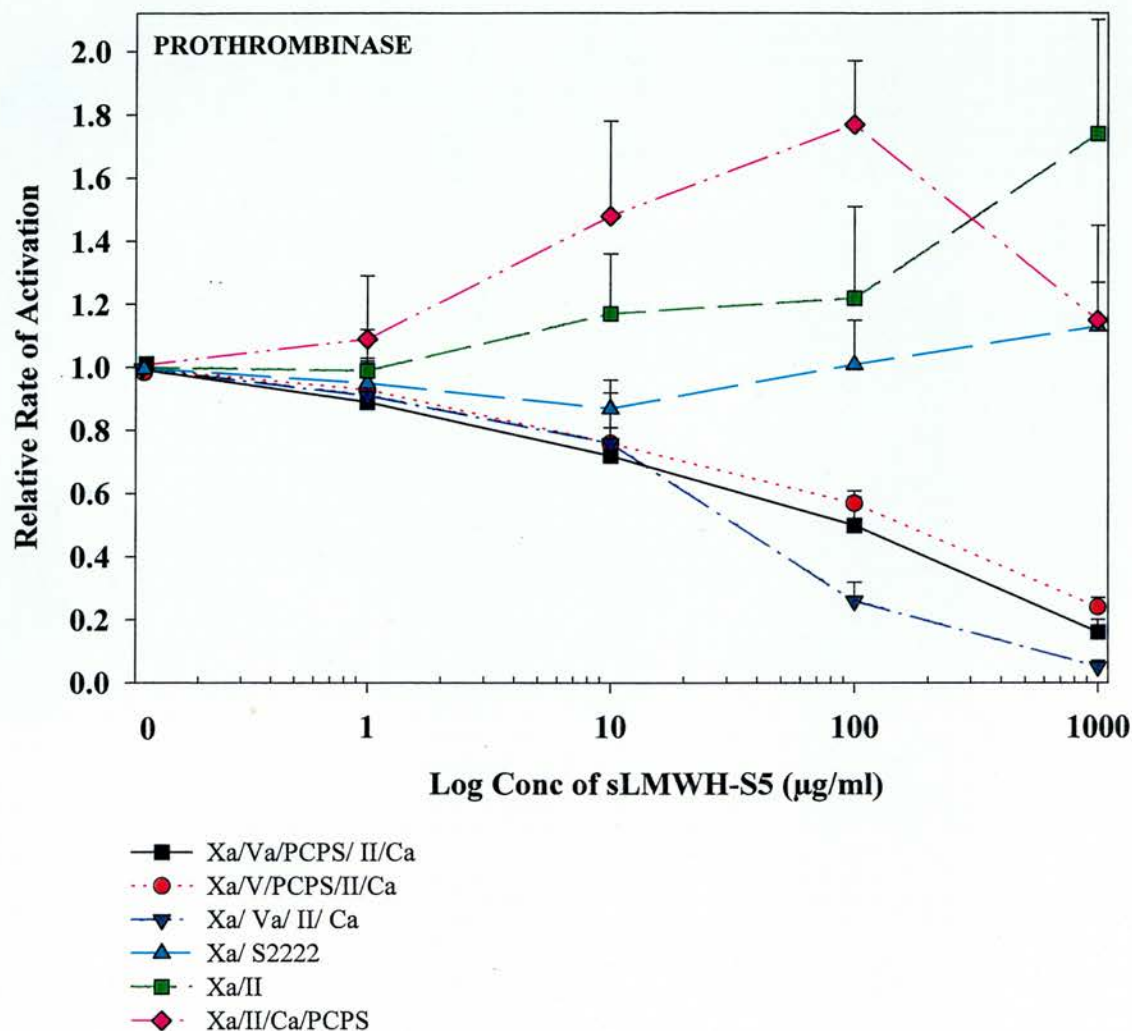
Figure 14 : Effect of hypersulphated LMWH (sLMWH) or heparin on the factor Xa clotting times in control plasma (●) or plasma immunodepleted of antithrombin. (■)

was possible to determine rates of activation under conditions where individual reactants were systematically removed or substituted. The influence of hypersulphated LMWH on the partially reconstituted activation complexes was used to reveal the component(s) responsible for glycosaminoglycan sensitivity. Figures 15 and 16 show the effects of the most highly sulphated LMWH, 'S5', on different combinations of reactants in prothrombinase and tenase systems, respectively. This particular hypersulphated LMWH was chosen as it demonstrated potent inhibitory effects on both intrinsic tenase and prothrombinase activity (see Table III).

A similar pattern of inhibition by LMWH "S5" was demonstrated in both prothrombinase and tenase systems. In systems consisting of the fully assembled enzyme complexes, a dose dependent inhibitory effect was present. In systems devoid of phospholipid, inhibitory effects were also evident in assays of tenase and prothrombinase, and similar  $IC_{50}$  values were found to those obtained with the complete enzyme complex. The  $IC_{50}$  for the effect of LMWH 'S5' on the full prothrombinase complex was  $30\mu\text{g/ml}$ , and was unchanged in a system devoid of phospholipid. For intrinsic tenase, the  $IC_{50}$  of LMWH was  $0.4\mu\text{g/ml}$ , and for a system devoid of phospholipid was  $0.5\mu\text{g/ml}$ . These findings indicate that the inhibitory effect is not phospholipid-dependent.

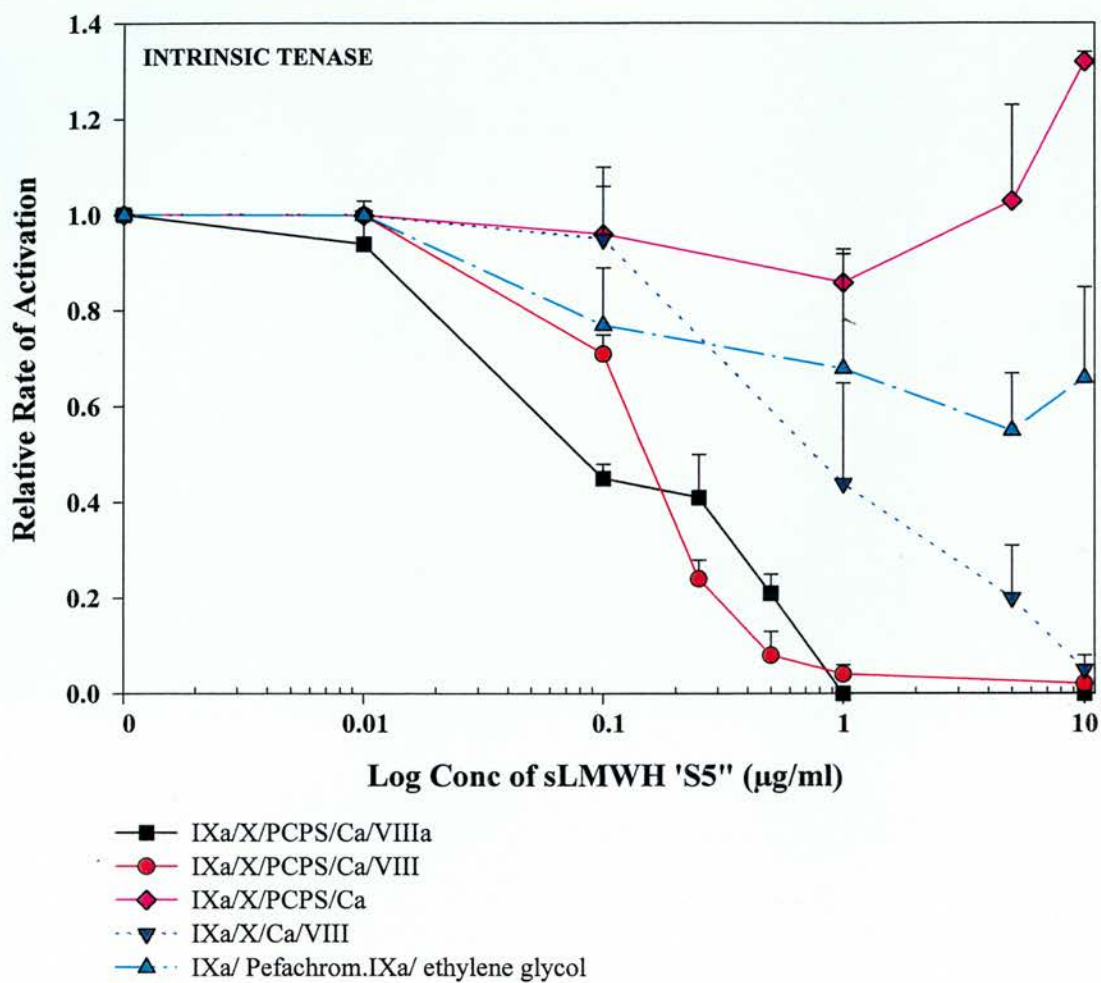
In systems devoid of cofactor alone (either fV/VIII or fVa/VIIIa), LMWH "S5" showed no inhibitory effect. For prothrombinase, it was possible to monitor thrombin generation in a system devoid of cofactor and phospholipid, as well as in a system devoid of cofactor, phospholipid and calcium; no inhibitory effect was seen in either system. For





**Figure 15 : Mechanism of Inhibition of Prothrombinase by sLMWH :**

sLMWH 'S5' was added at the indicated concentrations and prothrombinase activity was determined in a series of buffer systems in which individual components of prothrombinase were substituted or removed. Final reactant conditions are given in Section 2.2.3.



**Figure 16 : Mechanism of Inhibition of Intrinsic Tenase by sLMWH :**

sLMWH 'S5' was added at the indicated concentrations and the activation of fX was determined in a series of buffer systems in which individual components of tenase were substituted or removed. Final conditions for each system are given in Section 2.2.5.

intrinsic tenase, despite increases in reactant concentrations, rates of activation remained slow in a system devoid of fVIII and PCPS, and were unmeasurable in a system devoid of factor VIII, PCPS and calcium; in the latter system, even prolonged incubation of 3 hours failed to reveal any activation of fX (400nM) by fIXa (1 $\mu$ M).

In systems involving the substitution of non-activated for activated cofactor (fV or fVIII in place of fVa or fVIIIa), an initial lag phase was seen on graphs of thrombin and fXa generation vs time, reflecting the positive feedback effect of thrombin and fXa on fV or fVIII activation, respectively. The linear part of the graph was analysed to give an apparent rate of activation. Results showed a similar inhibitory effect to buffer systems using fVa or fVIIIa, indicating that activation of the cofactors have taken place. By inference, this suggests that the inhibitory steps are not occurring at the stage of cofactor activation.

For both systems, the effects of hypersulphated LMWH on the chromogenic activity of the enzymes were also examined. For prothrombinase, the effect of LMWH "S5" on the hydrolysis of the chromogenic substrate, S2222, by fXa, showed no evidence of inhibition.

With the system involving hydrolysis of Pefa IXa/60% ethylene glycol by fIXa alone, hypersulphated LMWH caused minor inhibition. However, the latter assay was of limited value since fIXa has virtually negligible activity with chromogenic amide substrates (Stürzebecher et al., 1997a)(Stürzebecher et al., 1997b)(Bode et al., 1997)(Brandstetter et al., 1995).

It is apparent that to maintain similar rates of activation, each system required differing final reactant concentrations and sampling times. However, the fact that similar

IC<sub>50</sub> values were obtained with or without phospholipid suggests that the effect of S5 was not limited to only the fully assembled activation complexes. This legitimises the approach of studying incompletely assembled activation complexes. Taking this into account, both intrinsic tenase and prothrombinase complexes show a phospholipid-independent, cofactor-dependent mechanism of inhibition by hypersulphated LMWH.

### 3.4. Determination of Mechanism of Inhibition by Kinetic Parameters :

Since the previous results suggest that hypersulphated LMWH disrupts assembly of the activation complexes, it was of interest to determine the mechanism of inhibition by detailed kinetic analysis. This is warranted because complex assembly affects both the  $k_{cat}$  and  $K_m$  of prothrombinase (Mann, 1987)(Nesheim, 1984)(Rosing et al., 1980). The increase in  $k_{cat}$  in both prothrombinase and intrinsic tenase has been attributed to the effect of the cofactor, whereas the phospholipid component of the enzyme complexes is responsible for the  $K_m$  effect. Thus, the turnover number,  $k_{cat}$  and the Michaelis constant,  $K_m$ , of the fXa/fVa complex for prothrombin, and of the fIXa/fVIIIa complex for fX were determined kinetically, in the presence of a saturating concentration of phospholipid (24-50 $\mu$ M), and in the absence or presence of hypersulphated LMWH.

*Determination of the Turnover Number for Prothrombinase Under Changing Reactant Conditions :* Prothrombinase activity was monitored under a series of differing experimental conditions to determine the optimal assay conditions for the determination of  $K_m$  and  $k_{cat}$  values. Results are summarised in Table IV.

Table IV : Summary of the “turnover number” derived from spectrophotometric assays of prothrombinase activity carried out under a series of varying experimental conditions. The turnover number given in panel 8 is consistent with values quoted in the literature (Krishnaswamy, 1990) under comparable experimental conditions.

	1	2	3	4	5	6	7	8	9	10	11
[Val]nM	0.2nM	5nM	20nM	20nM	20nM	20nM	20nM	20nM	20nM	20nM	20nM
[Xa]nM	0.5nM	0.05nM	0.05nM	0.05nM	0.5nM	0.05nM	0.05nM	0.05nM	0.05nM	0.05nM	0.05nM
[III]μM	0.6μM	2.4μM	2.4μM	2.4μM	2.4μM	2.4μM	2.4μM	2.4μM	2.4μM	2.4μM	2.4μM
[PCPS]μM	6μM	24μM	24μM	24μM	24μM	24μM	24μM	24μM	24μM	24μM	24μM
[Ca <sup>2+</sup> ]mM	2mM	8mM	8mM	8mM	8mM	8mM	8mM	8mM	8mM	8mM	8mM
chrom. substrate	0.11mM ChZThr	0.22mM ChZTh	0.44mM ChZThr	0.4mM S2444	0.4mM S2444	0.4mM S2444	0.4mM S2444	0.4mM S2444	0.44mM ChZ TPA	0.4mM S2444	0.4mM S2444
temp	RT	RT	RT	RT	RT	RT	RT	37°C	RT	RT	RT
-	-	-	-	-	-	-	1% Tween	-	-	-	-
turnover number nM IIa/sec/nMXa	0.267	1.78	5.297	13.3	12.57	13.84	14.16	21	7.05	7.69	2.18
GAG										100μg V20S	1000μg V20S

A turnover number of  $21\text{s}^{-1}$  was obtained in a spectrophotometric assay of prothrombinase, under the conditions outlined in panel 8 in Table IV, a value consistent with those quoted in the literature under comparable conditions (Krishnaswamy, 1990b). At room temperature, and under the same final reactant conditions, a turnover number of  $13\text{ s}^{-1}$  was obtained (panel 4, Table IV).

For practical reasons, the assay was adapted for use in the plate reader by adjusting the final volume. Turnover numbers of  $13.8\text{s}^{-1}$  and  $12\text{s}^{-1}$  were obtained under the same conditions (fVa 20nM, fXa 0.05nM, II 2.4 $\mu\text{M}$ , PCPS 24 $\mu\text{M}$ , calcium 8mM, S2444 0.4mM) for plate reader and spectrophotometric analysis, respectively.

Turnover numbers, expressed with respect to the limiting reactant component, were determined in the absence and presence of increasing concentrations of sLMWH (see figure 17a). A dose-dependent inhibitory effect was evident, and showed a pattern consistent with the inhibitory effect seen in earlier discontinuous assays. The  $\text{IC}_{50}$  value was found to be 226 $\mu\text{g/ml}$ , consistent with an  $\text{IC}_{50}$  value of 300 $\mu\text{g/ml}$  determined in a discontinuous assay. At concentrations of up to 1000 $\mu\text{g/ml}$ , thrombin generation was not completely inhibited. Higher sLMWH concentrations were not examined as there was the possibility of precipitation of glycosaminoglycan with chromogenic substrate.

*Determination of the Turnover Number for Intrinsic Tenase at Increasing Concentrations of sLMWH* : A turnover number of 17.9nM Xa/sec/nMVIIIa for intrinsic tenase was obtained at room temperature using a plate reader assay, in the absence of sLMWH. This result is consistent with reported turnover numbers determined under similar experimental

conditions in the literature (Chang et al., 1997).

With increasing concentrations of sLMWHs, a dose-dependent inhibitory effect was evident, and an  $IC_{50}$  value of  $0.37\mu\text{g/ml}$  was determined; this value is in agreement with an  $IC_{50}$  value of  $0.1\mu\text{g/ml}$  determined in a discontinuous assay. At concentrations of over  $10\mu\text{g/ml}$  of sLMWH, the rate of generation of fXa was almost completely inhibited (see figure 17b).

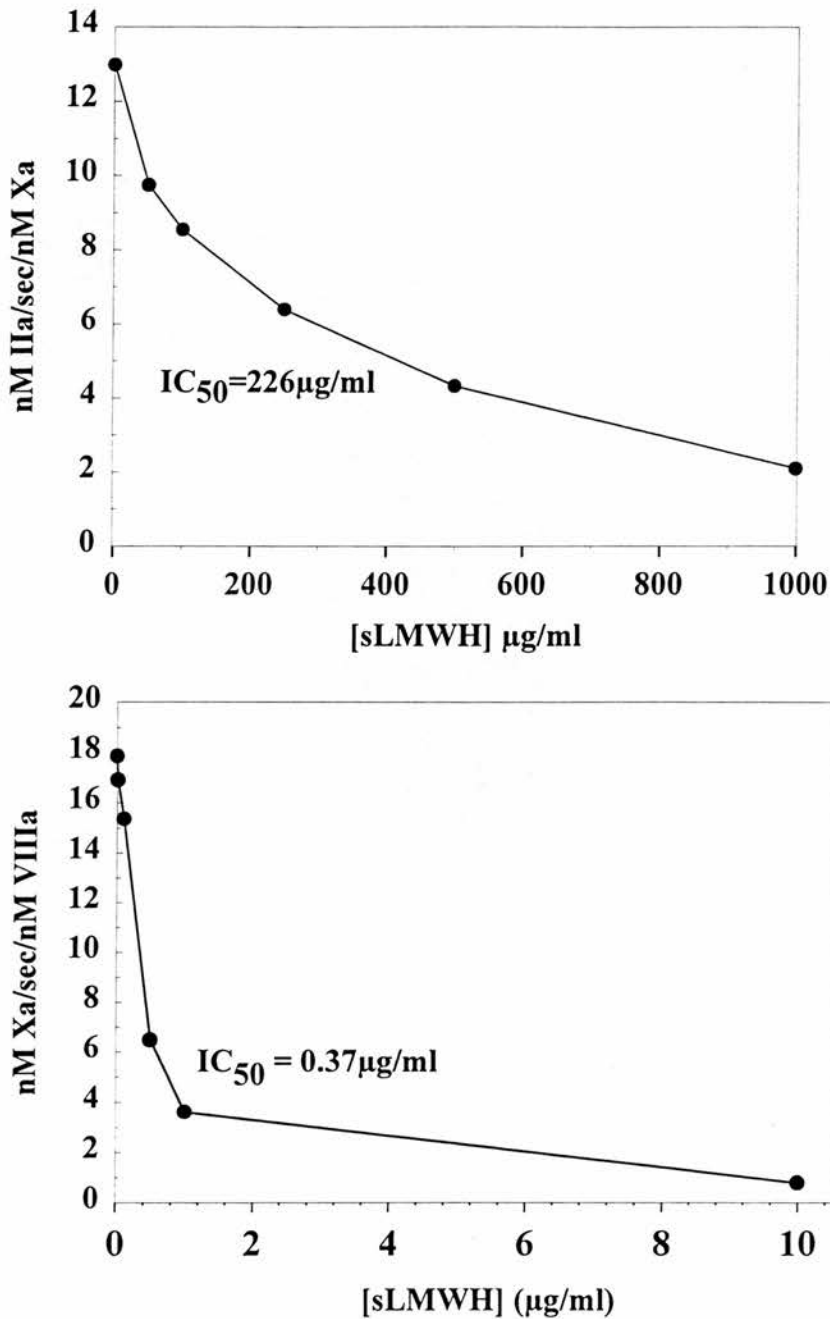
In conclusion, these continuous assays of prothrombinase and intrinsic tenase have demonstrated a dose-dependent inhibitory effect of the sLMWH, consistent with potencies seen in earlier discontinuous assays. In addition, in the presence of increasing concentrations of sLMWH, a differential inhibitory effect is seen, with almost complete inhibition of fXa generation.

#### *Determination of the $K_m$ for Prothrombinase and Intrinsic Tenase :*

The Michaelis constants for prothrombinase, and for intrinsic tenase, a measurement of the affinity of the substrate towards the enzyme complex, were determined by measuring the turnover numbers at a range of increasing substrate concentrations. The mean, standard deviation and individual  $K_m$  values for duplicate assays in the absence and presence of  $500\mu\text{g/ml}$  V20S (prothrombinase) and  $0.25\mu\text{g/ml}$  and  $0.5\mu\text{g/ml}$  V20S (intrinsic tenase) are shown in Table V, and the results for representative assays are shown in figures 18a and 18b.

For prothrombinase, in the presence of  $500\mu\text{g/ml}$  sLMWH, the  $k_{\text{cat}}$  was reduced by 3-fold whereas the  $K_m$  was unchanged at  $0.15\mu\text{M}$ , and the catalytic efficiency



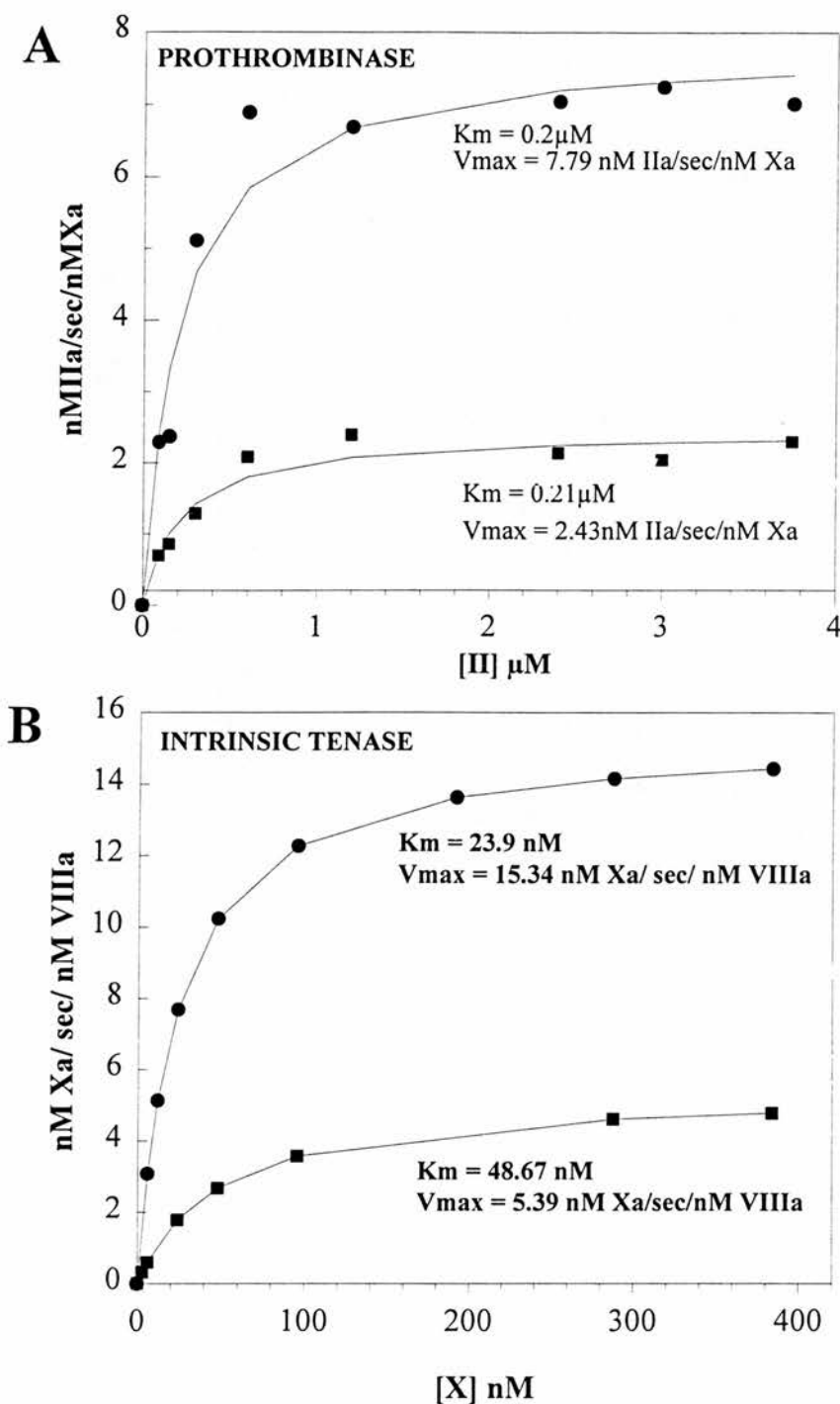


**Figure 17**

**PANEL A : Effect of sLMWH on the Turnover Number for Prothrombinase.** Turnover numbers were determined at the given concentrations of sLMWH. (2.4µM II, 0.05nM fXa, 20nM fVa). A dose-dependent inhibitory effect was seen.

**PANEL B : Effect of sLMWH on the Turnover Number for Intrinsic Tenase** Turnover numbers were determined at the given concentrations of sLMWH, under following conditions : fX 192nM, fIXa 2.5nM, fVIIIa 0.48nM, PCPS 24µM) A dose dependent inhibitory effect was present, and at concentrations of 10µg/ml, nearly complete inhibition of fXa generation occurred.





**Figure 18 :**

**PANEL A : Determination of  $K_m$  for Prothrombinase in Absence or Presence of sLMWH.**

The  $K_m$  for prothrombinase was determined by measuring the turnover number, expressed as nMIIa/s/nM Xa, at a range of increasing substrate concentrations, in the absence (●) and the presence (■) of 500  $\mu$ g/ml sLMWH. This graph represents the results of a single assay. The  $K_m$  remains unchanged in the presence of sLMWH, and  $k_{\text{cat}}$  is reduced 3-fold.

**PANEL B : Determination of  $K_m$  for Intrinsic Tenase in Absence or Presence of sLMWH.**

The  $K_m$  for intrinsic tenase was determined by measuring the turnover number, expressed as nMXa/sec/nMVIIa, at a range of increasing substrate concentrations, in the absence (●) and presence (■) of 0.25  $\mu$ g/ml sLMWH. This graph shows turnover numbers and derived  $K_m$  values for a single assay. The  $K_m$  is relatively unchanged and the  $k_{\text{cat}}$  is reduced 3-fold.

**TABLE V : Km VALUES for PROTHROMBINASE and INTRINSIC TENASE**

This table shows the absolute Km values for duplicate assays ("assay 1" and "assay 2") of prothrombinase (II 2.4μM, fVa 20nM, fXa 0.05nM, PCPS 24μM, CaCl<sub>2</sub> 8mM, S2444 400μM) expressed in μM, and for intrinsic tenase (fX 192nM, fVIII/VIIIa 0.48nM, CaCl<sub>2</sub> 8mM, fIXa 2.5nM, ChZX 500μM), expressed in nM. Assays were performed in the absence and presence of the given concentrations of sLMWH.

1. Km Values for Prothrombinase Assays :

	no sLMWH		500μg/ml sLMWH	
	*assay 1	assay 2	assay 1	assay 2
Km (μM)	0.2	0.1	0.21	0.1
Vmax (nM IIa/s/nM Xa)	7.8	4.3	2.4	2.0
	mean Km 0.15μM SD 0.05		mean Km 0.15μM SD 0.05	

2. Km values for Intrinsic Tenase Assays :

	no sLMWH		500μg/ml sLMWH	
	assay 1	assay 2	assay 1	assay 2
Km (nM)	32.9	23.9	71.5	48.7
Vmax (nM IIa/s/nM Xa)	16.0	15.3	2.0	5.4
	mean Km 28.4nM SD 4.5		mean Km 60.1nM SD 11.4	

( $k_{cat}/K_m$ ) was reduced from a value of  $39\text{M}^{-1}\text{s}^{-1}$  to  $12\text{M}^{-1}\text{s}^{-1}$ . According to Michaelis-Menten kinetics, this pattern is consistent with a noncompetitive mechanism of inhibition. A similar pattern of inhibition was seen with intrinsic tenase; in the presence of  $0.5\mu\text{g/ml}$  sLMWH,  $k_{cat}$  was reduced 8-fold, and the  $K_m$  increased minimally from 28 to  $60\text{nM}$ . The catalytic efficiency was reduced from a value of  $0.56\text{M}^{-1}\text{s}^{-1}$  to  $0.07\text{M}^{-1}\text{s}^{-1}$ . These results are consistent with the noncompetitive inhibitory effect of unfractionated heparin on intrinsic tenase, demonstrated by Barrow et al. (Barrow et al., 1994a).

With minimal changes in  $K_m$ , and relatively greater effects on  $k_{cat}$ , it was important to determine the way in which the cofactor influences the enzyme complex in the presence of hypersulphated LMWH, and, for this reason, the effect of sLMWH on the apparent dissociation constant,  $K_{d_{app}}$  of fVa for prothrombinase was determined. The  $K_{d_{app}}$  of the enzymes, fXa for prothrombinase, and fIXa for intrinsic tenase were also determined.

*Determination of  $K_{d_{app}}$  of enzyme and cofactor to prothrombinase and intrinsic tenase:*

The  $K_{d_{app}}$  of fVa for prothrombinase was determined kinetically by monitoring the concentration dependence of cofactor on substrate activation, in the presence of a saturating concentration of phospholipid. In the presence of  $500\mu\text{g/ml}$  sLMWH, the  $K_{d_{app}}$  of fVa for prothrombinase increased from a mean value of  $1.18\text{nM}$  to  $9.96\text{nM}$ , and in an analogous experiment, determination of the  $K_{d_{app}}$  for fXa, determined by monitoring the concentration dependence of enzyme on substrate activation, also increased, from a mean value of  $0.18\text{nM}$  to  $4.7\text{nM}$  (see Table VI, and figures 19a and b).

The  $K_{d_{app}}$  of fIXa for intrinsic tenase was determined in an analogous set of assays, and

**TABLE VI : SUMMARY of KINETIC PARAMETERS**

Panel 1 shows the absolute  $K_{d_{app}}$  values (nM) for duplicate assays ("assay1" and "assay2") of fVa for prothrombinase (II 2.4 $\mu$ M, fVa 0 - 20nM, fXa 0.05nM, PCPS 24 $\mu$ M, CaCl<sub>2</sub> 8mM, S2444 400 $\mu$ M), and panel 2 shows the  $K_{d_{app}}$  values of fXa for prothrombinase (II 2.4 $\mu$ M, fXa 0 - 25nM, fVa 2nM, PCPS 24 $\mu$ M, CaCl<sub>2</sub> 8mM, S2444 400 $\mu$ M), in the absence or presence of the given concentration of sLMWH. The values for the  $K_{d_{app}}$  of fIXa (nM) for intrinsic tenase (fX 192nM, fVIII/VIIIa 0.48nM, CaCl<sub>2</sub> 8mM, fIXa 0 -4nM, ChZX 500 $\mu$ M), in the absence or presence of the given concentration of sLMWH, are shown in Panel 3.

1.  $K_{d_{app}}$  of fVa towards Prothrombinase :

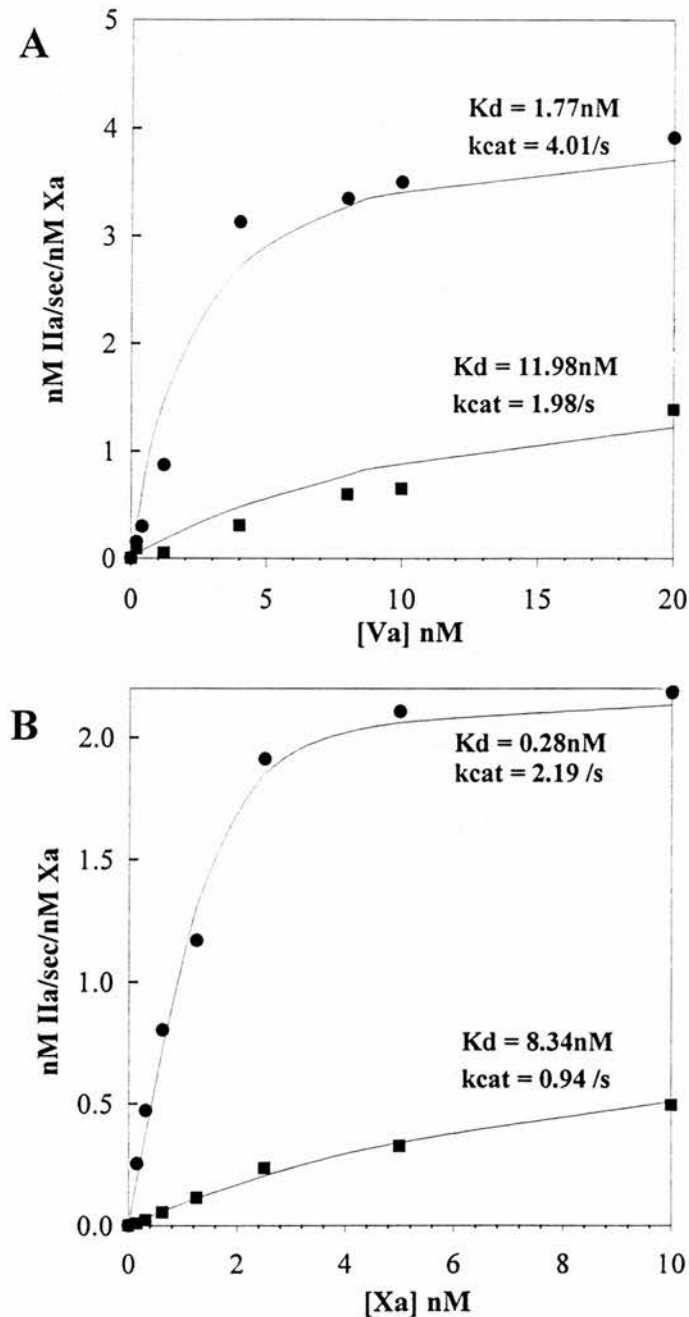
	<u>no sLMWH</u>		<u>500µg/ml sLMWH</u>	
	assay 1	assay 2	assay 1	assay 2
Kd (nM)	1.77	0.63	11.98	7.34
k <sub>cat</sub> (nMIIa/s/nM Xa)	4.01	4.52	1.98	2.45
mean Kd : 1.2		mean Kd : 9.96		
SD : 0.57		SD : 2.32		

2.  $K_{d_{app}}$  of fXa towards Prothrombinase :

<u>no sLMWH</u>		<u>500µg/ml sLMWH</u>		
	assay 1	assay 2	assay 1	assay 2
Kd	0.28	0.09	8.34	1.17
k <sub>cat</sub> (nM IIa/s/nM Xa)	2.19	1.24	0.94	0.39
mean Kd : 0.18		mean Kd : 4.75		
SD : 0.09		SD : 3.58		

3.  $K_{d_{app}}$  of fIXa towards Intrinsic Tenase :

	<u>no sLMWH</u>		<u>0.25µg/ml sLMWH</u>	
	assay 1	assay 2	assay 1	assay 2
Kd (nM)	1.93	1.27	3.13	2.8
k <sub>cat</sub> nMXa/s/nM VIIIA	17	11.56	4.56	4.7
mean Kd : 1.6		mean Kd : 2.96		
SEM : 0.33		SEM : 0.16		



**Figure 19 :**

**PANEL A. Determination of  $K_{d_{app}}$  of fVa for Prothrombinase in Absence or Presence of sLMWH.**

The  $K_{d_{app}}$  of fIXa for prothrombinase was determined by measuring the concentration dependence of cofactor, fVa on substrate activation. The graph shows turnover numbers (nMIIa/s/nMXa), at a range of increasing cofactor concentrations, in the absence (●) or the presence (■) of 500  $\mu$ g/ml. This graph represents the results of a single assay.

**PANEL B. Determination of  $K_{d_{app}}$  of fXa for Prothrombinase in Absence or Presence of sLMWH.**

The  $K_{d_{app}}$  of fXa for prothrombinase was determined in an analogous set of assays. This graph shows turnover numbers and derived kcat and Kd parameters for a single assay.

remained relatively unchanged in the presence of 0.25 µg/ml sLMWH (see figure 20).

The interpretation of this data is not clear; an increase in the  $K_{d_{app}}$  of fVa, and of fXa towards prothrombinase suggests individually a change in the affinity of enzyme, and of cofactor, towards the assembled complex in the presence of sLMWH. However, the  $K_{d_{app}}$  for intrinsic tenase remains relatively unchanged in the presence of sLMWH.

### **3.5. Effect of Hypersulphated LMWH on Prothrombinase and Intrinsic Tenase in an Assay Using Platelets as the Membrane Surface :**

Although PCPS vesicles are used experimentally as an analogue of thrombin-stimulated platelets, for several reasons, synthetic PCPS vesicles do not completely mimic the platelet surface (London and Walsh, 1996). These include differences in electrostatic and hydrophobic interactions between cofactor, enzyme and negatively charged lipid surfaces (Kalafatis et al., 1994)(Gilbert and Baleja J., 1995)(London and Walsh, 1996)(London et al., 1996). Differences also exist in prothrombinase complex assembly on the two surfaces (Larson et al., 1998). A series of assays were carried out substituting synthetic isolated washed human platelets for synthetic PCPS vesicles, to determine if this would influence the potent inhibitory effect already demonstrated on the PCPS vesicle surface.

In a series of assays of intrinsic tenase activity using both activated (calcium ionophore, A23187) and non-activated human platelets, a dose-dependent inhibition of fXa generation was observed (see figure 21a), with a mean  $IC_{50}$  value of 1 µg/ml. In addition, a dose-dependent increase in the "lag phase" prior to the initiation of the clotting process was

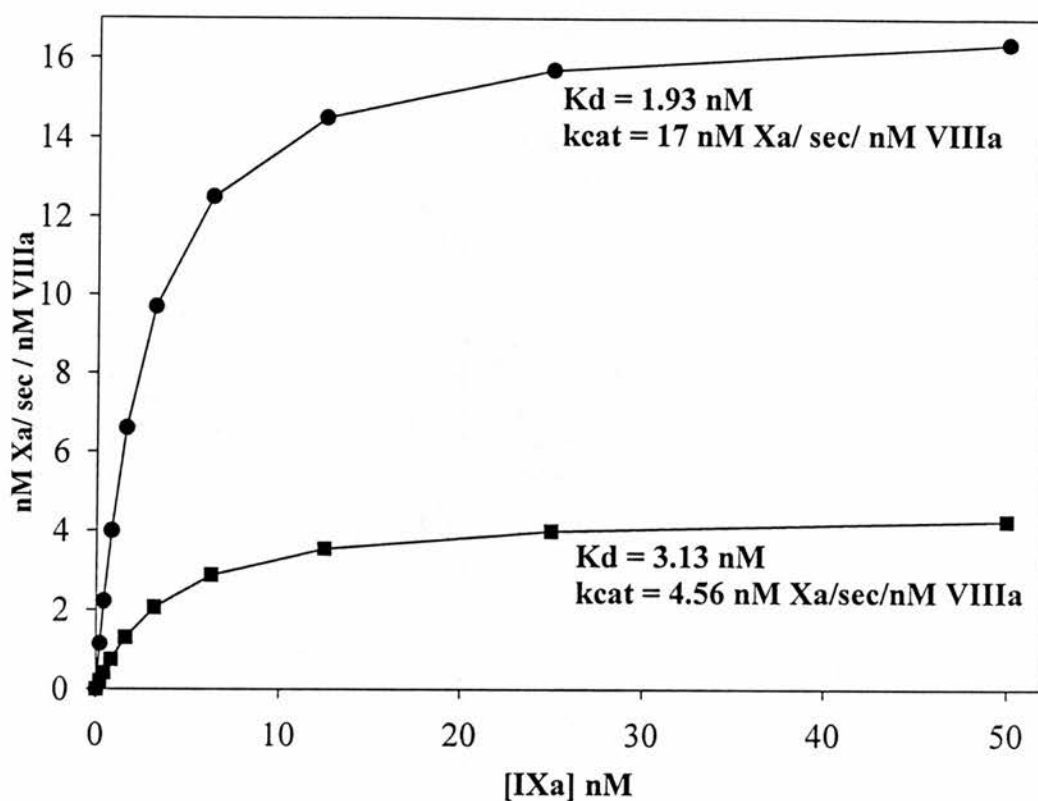


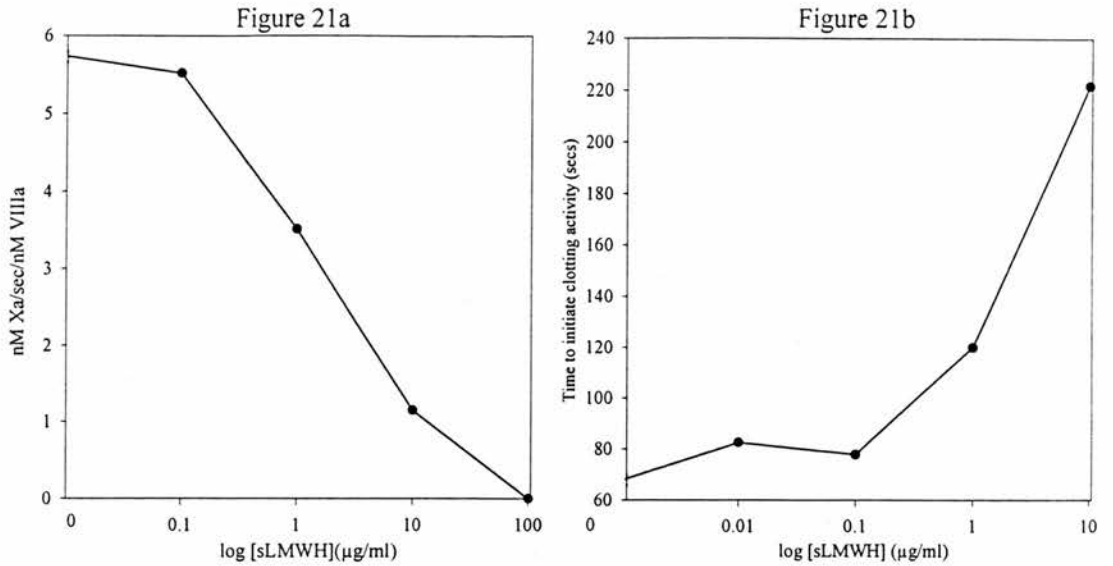
Fig. 20 : Determination of the Apparent  $K_d$  for fIXa towards Intrinsic Xase

The  $K_{d_{app}}$  for fIXa towards the assembled intrinsic tenase complex was determined kinetically by monitoring the concentration dependence of enzyme on substrate activation. The catalytic rates of intrinsic Xase were determined in the absence (●) and presence (■) of  $0.25\mu\text{g/ml}$  sLMWH, a concentration that was higher than the  $IC_{50}$  value.

observed, starting at a concentration of  $0.1\mu\text{g/ml}$  (see figure 21b). For prothrombinase, using the same isolated activated platelet preparation as in the intrinsic tenase assays, and in the presence of exogenous fVa, no inhibition was evident. In a repeat set of prothrombinase assays, where exogenous fVa was omitted (Mast and Broze, Jr., 1996), inhibition of thrombin generation was seen at sLMWH concentrations of  $1000\mu\text{g/ml}$ , and a dose-dependent prolongation of the "lag-phase" was observed, starting at a concentration of  $10\mu\text{g/ml}$  (see figures 22a and 22b).

These results demonstrate that despite differences in enzyme complex assembly and catalytic function between synthetic PCPS vesicles and platelets, hypersulphated LMWH inhibits the activity of prothrombinase and intrinsic tenase on both membrane surfaces.

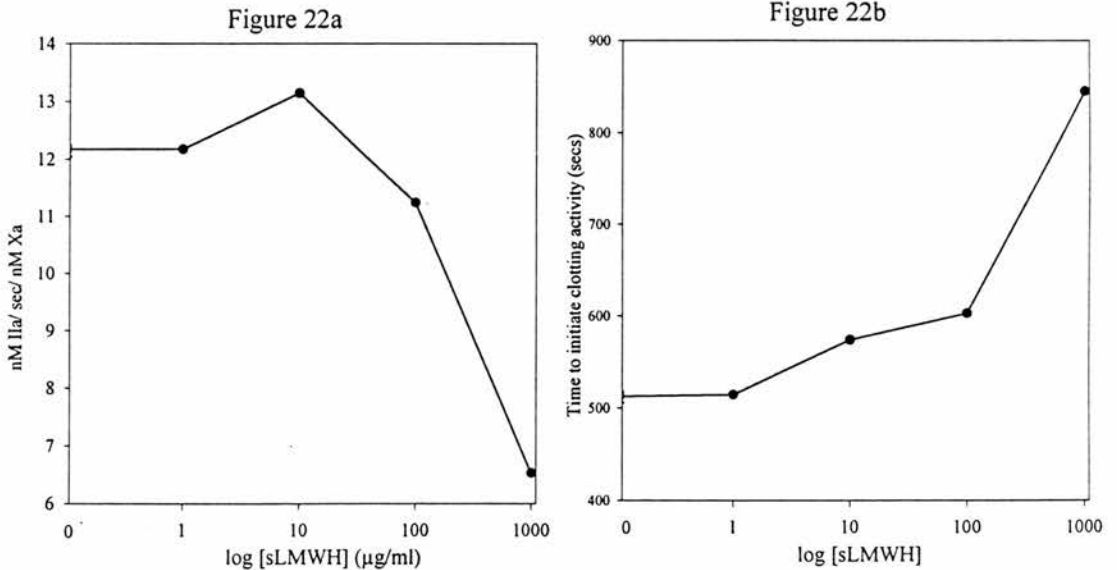




**Figure 21 : Effect of sLMWH on Intrinsic Tenase in an Assay Substituting PCPS Vesicles for Isolated Washed Human Platelets.**

Figure 'a' shows the effect of sLMWH on the turnover number of intrinsic tenase, at room temperature and represents the results of a single assay.

Figure 'b' shows the effect of sLMWH on the time to initiate generation of fXa, and this graph also represents data from one assay.



**Figure 22 : Effect of sLMWH on Prothrombinase in an Assay Substituting PCPS Vesicles for Isolated Washed Human Platelets.**

Figure 'a' shows the effect of sLMWH on the turnover number of prothrombinase, at room temperature. (The conditions were 2.4μM II, 0.05nM fXa,  $9.2 \times 10^9$  platelets/ml, no fVa, calcium 4mM). This graph represents the results of a single assay. Figure 'b' shows the effect on the time to initiate generation of thrombin (secs), and also shows data from a representative assay.

## **CHAPTER 4 : DISCUSSION**

### **4.1. Charge-dependent Mechanism of Inhibition**

#### **4.2.1 Effect of Sulphated Glycosaminoglycans on Blood Coagulation**

### **4.2. Cofactor-dependent, Phospholipid-independent Mechanism of Inhibition**

### **4.3. Model for the Mechanism of Inhibition of Prothrombinase and Intrinsic Tenase by Hypersulphated LMWH**

### **4.4. Future Directions**

#### **4.4.1. in vitro Studies**

Determination of Effect of sLMWH on Enzyme-Cofactor Interaction

Determination of Effect of sLMWH on Cellular Surfaces

#### **4.4.2. in vivo Studies : Animal Models of Arterial Thrombosis**

### **4.5. Summary and Perspectives**

## CHAPTER 4 : DISCUSSION

The intrinsic tenase and prothrombinase complexes contribute directly to thrombin generation in the process of blood coagulation, and as such, are ideal targets for the inhibition of blood coagulation. Since these multicomponent complexes are assembled from intrinsic and activated components, there are numerous approaches available for their inhibition. These include direct inactivation of the enzyme or disruption of the capacity of the complex to assemble productively. Inhibitors available to date include active site-blocked factors IXa (IXa<sub>i</sub>) and Xa (Xa<sub>i</sub>) (Benedict et al., 1991; Wong et al., 1997), anti-fIX/IXa monoclonal antibody (Feuerstein et al., 1997), and active site-directed fXa inhibitors (Tuszynski et al., 1987; Vlasuk, 1993; Cappello et al., 1995; Hara et al., 1995; Taniuchi et al., 1998).

Active site-blocked fXa and fIXa competitively inhibit prothrombinase and intrinsic tenase in vitro (Wong, Gunn et al., 1997), and effectively inhibit thrombosis in several animal models of venous (Wong, Gunn et al., 1977) and arterial thrombosis (Benedict, Ryan et al., 1991)(Benedict, Ryan et al., 1993)(Spanier, Oz et al., 1997). Active site blocked fXa inhibits the assembly of activated enzyme within prothrombinase (Nesheim, Kettner et al., 1981)(Skogen, Esmon et al., 1984), and may also substitute for the substrate, fX within intrinsic tenase. (Benedict, Ryan et al., 1993).

BC2, a murine anti-fIX/IXa monoclonal antibody, inhibits fIX conversion to fIXa, and has potent antithrombotic activity in a rat carotid artery thrombosis model (Feuerstein, Nichols et al., 1997).

Active-site directed inhibitors of fXa include the natural anticoagulants, tick

anticoagulant peptide (TAP) (Waxman, Smith et al., 1990)(Vlasuk, 1993), antistasin (Dunwiddie, Thornberry et al., 1989)(Tuszynski, Gasic et al., 1987), *Ancylostoma caninum* anticoagulant peptide (AcAP) (Cappello, Vlasuk et al., 1995), and synthetic inhibitors such as DX 9065a (Hara, Yokoyama et al., 1995)(Hara, Yokoyama et al., 1994) and YM 60828 (Taniuchi, Sakai, et al., 1998), exert their inhibitory effect by binding competitively and reversibly to fXa. In addition to inhibiting free fXa, direct inhibitors inactivate fXa bound to phospholipid surfaces (Krishnaswamy and Betz, 1997).

Heparin also inhibits intrinsic tenase (Barrow et al., 1994a); in plasma-free systems, both unfractionated heparin and low molecular weight heparins inhibit the activation of fX by a noncompetitive mechanism, but have no effect on prothrombinase at therapeutic concentrations. In plasma systems, this antithrombin-independent effect is largely overshadowed by the ability of heparin to catalyse the inhibition by antithrombin of thrombin and fXa (Hirsh, 1991), and to a much lesser extent, to catalyse the inhibition of thrombin by heparin cofactor II (Tollefsen, 1995).

To circumvent this problem we first chemically modified LMWH to reduce its affinity for antithrombin, and demonstrated that the modified LMWH retains its ability to inhibit intrinsic tenase. The aim of this project was to investigate the mechanism by which LMWH inhibits these coagulation complexes so as to exploit these properties in the development of a new inhibitor.

From the results given in Chapter 3, it was shown that hypersulphation of LA-LMWH produces significant increases in the potency of the heparin. Hypersulphated

LMWH was subsequently used to investigate the mechanism of inhibition of prothrombinase and intrinsic tenase. The results revealed that the inhibition of intrinsic tenase and prothrombinase by hypersulphated low molecular weight heparin is:-

- a) Charge-dependent
- b) Antithrombin-independent
- c) Cofactor-dependent
- d) Phospholipid-independent, and
- e) Non-competitive

#### **4.1. Charge-dependent Mechanism of Inhibition :**

Periodate oxidation, a technique which selectively cleaves bonds of non-sulphated uronic acid residues (Young and Hirsh, 1990), and borohydride reduction were used to modify LMWH so as to lower its antithrombin affinity by 1500-fold. Despite eliminating the antithrombin-dependent properties of the LMWH, preliminary studies in buffer systems demonstrated residual anticoagulant activity caused by the direct inhibition of intrinsic tenase. Using this LA-LMWH, LMWH was N-desulphated using a solvolytic desulphation technique (Inoue and Nagasawa, 1976).

At fixed concentrations of fIXa, fVIIIa, fX and PCPS vesicles, LA-LMWH caused 50% inhibition of the initial velocity of fX activation at a concentration of 16.3 $\mu$ g/ml. Enoxaparin, a LMWH with normal antithrombin affinity, inhibited intrinsic tenase to the same extent, with an  $IC_{50}$  of 13.2 $\mu$ g/ml. In contrast, N-desulphated LMWH had less inhibitory potency, and a mean  $IC_{50}$  of 166 $\mu$ g/ml was obtained. These findings indicated

that the inhibition of intrinsic tenase was antithrombin-independent, but charge-dependent, and raised the possibility that hypersulphation of LMWH might increase the inhibitory potency. To test this hypothesis, LA-LMWH was hypersulphated using sulphur trioxide as a sulphate donor. A series of LMWHs of increasing degrees of sulphation, and increasing degrees of negative charge, were examined in assays of intrinsic tenase activity. The results indicated that progressive hypersulphation of LMWH increases the inhibitory potency of the glycosaminoglycan, reflected by progressive reduction in the  $IC_{50}$  values. The most highly sulphated LMWH, "S5" was found to be a 32-fold more potent inhibitor of intrinsic tenase than LMWH, with an  $IC_{50}$  value of  $0.47\mu\text{g/ml}$ . In addition, increasing the negative charge of the glycosaminoglycan introduced prothrombinase inhibitory activity. The inhibitory effects of our series of hypersulphated LMWHs were also examined in assays of prothrombinase in buffer systems. A similar charge-dependent inhibitory effect was found, with progressive hypersulphation of LMWH increasing the degree of inhibition, reflected by reduction in the  $IC_{50}$  values. The  $IC_{50}$  values of hypersulphated LMWHs for prothrombinase were 2 orders of magnitude higher than those for intrinsic tenase ( $IC_{50}$  values for "S5" of  $0.47\mu\text{g/ml}$  (intrinsic tenase) and  $30\mu\text{g/ml}$  (prothrombinase)), demonstrating that the most potent inhibitory effect occurs towards the generation of fXa by intrinsic tenase.

In plasma-based coagulation assays, hypersulphated LMWH demonstrated a similar AT-independent inhibitory effect towards both intrinsic tenase and prothrombinase. The APTT was used to reflect activity of intrinsic tenase and

prothrombinase, and the fXa-clotting time was used as a measure of prothrombinase activity. Both APTT and fXa-clotting times were prolonged in antithrombin-depleted and control plasma to the same extent. In contrast, unfractionated heparin caused no prolongation of either clotting time in plasma immunodepleted of antithrombin. The plasma-based assays demonstrate that the inhibitory effects of hypersulphated LMWHs towards both intrinsic tenase and prothrombinase seen in buffer systems also occur within plasma systems.

#### **4.1.1. Effect of Sulphated Glycosaminoglycans on Blood Coagulation :**

Less sulphated polysaccharides such as chondroitin sulphate, dermatan sulphate, keratan sulphate and hyaluronic acid were observed to have no inhibitory effect on intrinsic tenase (Barrow et al., 1994a). More highly sulphated polysaccharides, such as dextran sulphate, possess an anticoagulant effect (Ricketts C.R., 1952); between 1-1.3 sulphate groups per glucose unit are required to cause maximum anticoagulant activity. Similarly, sulphation of the natural polysaccharide, 'curdlan', creates active  $\beta$ -1,3-glucan sulphates, which produce a concentration dependent effect in APTT and thrombin time assays (Alban et al., 1995). Additionally, Tiozzo et al. have demonstrated in an APTT assay that N-desulphated heparins have a reduced anticoagulant effect (Tiozzo et al., 1993). It was also demonstrated that as the degree of O-desulphation increases, both anticoagulant and antiproliferative effects (in baby hamster kidney and smooth muscle cell lines) decrease. Likewise, unfractionated heparin, LMWH, pentosanpolysulphate (a semi-synthetic sulphated polysaccharide), and lactobionic acid (a synthetic polysaccharide) suppress the activity of natural killer

cells, decrease lymphocyte cell size, alter cell surface structure at clinically relevant dosages, and may interfere with the tumour killing process (Johann et al., 1995). Thus, hypersulphated LMWHs may have additional clinical benefits, for example in the treatment of thrombosis in patients undergoing management of malignant conditions.

#### **4.2. Cofactor-dependent, Phospholipid-independent Mechanism of Inhibition**

Prothrombinase and intrinsic tenase are multicomponent enzyme complexes; taking into account the catalytic advantage conferred when all the components of the enzyme complex are present (Mann, 1987; Mann et al., 1988), it was possible to determine rates of activation under conditions where individual components were systematically substituted or removed. The effect of the most potent hypersulphated LMWH, 'S5', as examined on the partially reconstituted activation complexes, to investigate the components necessary for glycosaminoglycan sensitivity.

For both prothrombinase and intrinsic tenase, similar patterns of inhibition were evident. In systems devoid of phospholipid, similar inhibitory effects were found to those obtained with the complete enzyme complex, reflected by comparable  $IC_{50}$  values between the two systems. These findings indicated that the inhibitory effects were not dependent on the presence of phospholipid.

To investigate these findings further, assays were carried out to determine the effect of hypersulphated LMWH on prothrombinase activity and intrinsic tenase activity using platelets in place of synthetic phospholipid vesicles.

Despite differences in the interaction of the components of prothrombinase and



intrinsic tenase and their assembly on platelets and phospholipids (Kalafatis et al., 1994; Gilbert and Baleja, J., 1995; London and Walsh, 1996), inhibition of the activity of these enzyme complexes by hypersulphated LMWH was still observed, irrespective of the membrane surface used.

#### **4.3. Proposed Model for the Mechanism of Inhibition of Prothrombinase and Intrinsic Tenase by Hypersulphated LMWH :**

The inhibition of both prothrombinase and intrinsic tenase by hypersulphated LMWH follows a "non-competitive" pattern of inhibition. A non-competitive inhibitor can bind to a site on the enzyme other than the active site, and may cause a conformational or deforming change in the enzyme such that the enzyme-substrate complex can no longer be formed at its normal rate. (Lehninger, 1975; Wood et al., 1981) Increasing the substrate concentration does not reverse the effect. The Michaelis constant,  $K_m$  is unaltered, and the  $V_{max}$  is reduced. This is in contrast to competitive inhibition, where the inhibitor binds at the same site (active site) of the substrate and competes with it. Under such circumstances, the  $K_m$  would be increased (increasing the substrate concentration would reverse the inhibitory effect) and the  $V_{max}$  would remain unaltered, as the inhibitor does not interfere with the rate of breakdown of the enzyme-substrate complex (Creighton, T.E., 1992). In this respect, hypersulphated LMWH inhibits intrinsic tenase in the same manner as unfractionated heparin, which also demonstrates a noncompetitive mechanism of inhibition in a purified buffer system (Barrow et al., 1994a).

In addition,  $k_{cat}$  was found to be reduced for both enzyme complexes in the presence of hypersulphated LMWH; the  $k_{cat}$  value reflects the rate of product formation by the enzyme-substrate complex and is proportional to the difference in free energy between the ground state (Michaelis complex) and the transition state (Chang et al., 1998).  $k_{cat}$ , referred to as the 'turnover number', reflects moles of substrate consumed per unit of time per mole of enzyme. For a multicomponent enzyme, such as intrinsic tenase or prothrombinase, the functional enzyme concentration is determined by the affinities and concentrations of the individual components. Where all other components are saturating, the limiting component reflects the enzyme concentration. Therefore,  $k_{cat}$  represents the number of times each enzyme molecule catalyses the reaction.

Determination of turnover numbers for prothrombinase and intrinsic tenase in the presence of increasing concentrations of hypersulphated LMWH indicated a dose-dependent inhibitory effect for both systems. In addition, there appears to be a differential inhibitory effect, with almost complete inhibition of fXa generation by intrinsic tenase, at concentrations of up to 10 $\mu$ g/ml. At concentrations of sLMWH above a therapeutic range (concentrations of >1000 $\mu$ g/ml), there is considerably reduced, yet ongoing thrombin generation by prothrombinase.

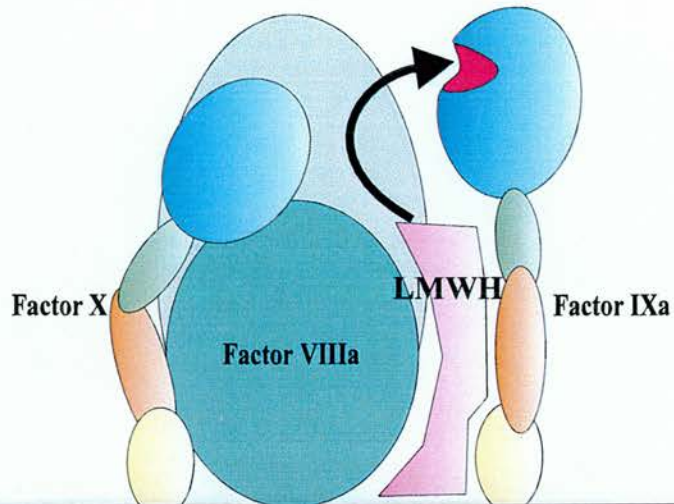
To further determine the mechanism of inhibition,  $K_m$  and  $k_{cat}$  of the fXa/fVa complex for prothrombin, and of the fIXa/fVIIIa complex for fX were determined kinetically in the absence and presence of sLMWH, at a concentration above the determined  $IC_{50}$  value. For prothrombinase, in the presence of 500 $\mu$ g/ml sLMWH,  $K_m$

was unchanged ( $0.15\mu\text{M}$ ), and  $k_{\text{cat}}$  was reduced 3-fold. This pattern is consistent with noncompetitive inhibition. A similar pattern of inhibition was seen with intrinsic tenase; in the presence of  $0.5\mu\text{g/ml}$ ,  $k_{\text{cat}}$  was reduced 8-fold, and the  $K_m$  increased minimally from 28 to  $60\text{nM}$ .

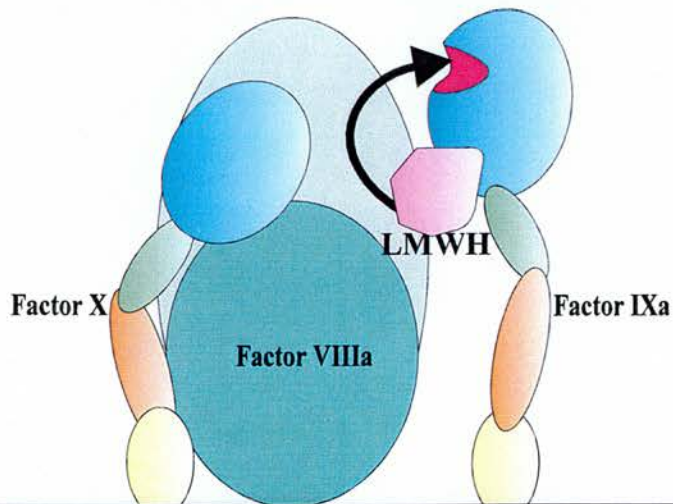
The  $K_{d_{\text{app}}}$ , a measure of the affinity of enzyme or cofactor towards the "substrate", in this case the affinity of the enzyme or the cofactor towards the rest of the assembled enzyme complex, was found to be increased for prothrombinase by one order of magnitude in the presence of hypersulphated LMWH, but was relatively unchanged for an analogous system with intrinsic tenase.

Brandstetter's model of intrinsic tenase (Brandstetter et al., 1995), proposes that the arched fIXa molecule lies across the cofactor, fVIIIa, allowing the binding of fX to the opposite side of fVIIIa, forcing fXa cleavage sites close to the substrate binding site of fIXa. Using this model to reflect intrinsic tenase and the analogous complex, prothrombinase, there are two possible mechanisms of enzyme complex disruption, which are schematically shown in figure 23.

a) Scheme 'A' (steric mechanism) involves disruption of the enzyme/cofactor interaction by hypersulphated LMWH, in turn causing changes in the amount of fIXa complexed to fVIIIa at a given concentration of proteins, reducing the effective enzyme concentration. This mechanism, consistent with a cofactor-dependent, phospholipid-independent, noncompetitive mechanism of inhibition would account for the reduction in  $k_{\text{cat}}$  and  $V_{\text{max}}$  seen with both assays of intrinsic tenase and prothrombinase in the

**SCHEME A**

sLMWH causes disruption of the enzyme-cofactor interaction, at one or several binding sites. This may reduce the effective enzyme concentration, or may cause impaired binding between the substrate and the enzyme active site.

**SCHEME B**

sLMWH binds to an exosite(s) on the enzyme causing impaired binding of the substrate. Binding to the exosite may also weaken the affinity of the enzyme and cofactor, and this process may or may not be involved in the inhibitory process.

**Figure 23 : Proposed Models for the Mechanism of Inhibition of Intrinsic Tenase by Hypersulphated LMWH.**

presence of hypersulphated LMWH, and also accounts for the increase in  $K_{d_{app}}$  of both enzyme and cofactor towards prothrombinase. The model does not, however, explain the lack of change in  $K_d$  with intrinsic tenase.

b) Scheme 'B' (allosteric mechanism) involves hypersulphated LMWH binding to an extended macromolecular binding site (exosite(s)) on the enzyme, causing impaired binding of substrate, and possibly also cofactor, with subsequent reduction in the rate of fX activation. This model is in agreement with the model of macromolecular substrate recognition proposed by Krishnaswamy and colleagues in 1997 (Krishnaswamy and Betz, 1997; Betz and Krishnaswamy, 1998).

In support of Scheme 'B', studies using fluorescent derivatives of fIXa have shown a direct effect of fVIIIa on the catalytic activity of the enzyme (Mutucumarana et al., 1992). The active site of fIXa is positioned more than 70Å above the phospholipid surface; binding of fVIIIa does not alter the distance of fIXa from the surface, but does alter both the emission intensity and anisotropy of fluorophore-labeled active site, suggesting that cofactor stimulation of fIXa catalytic activity results, at least in part, by conformational change in the active site when fVIIIa binds to the enzyme on the phospholipid surface (Mutucumarana et al., 1992). This provides evidence that disruption of enzyme and cofactor at sites away from the active site could change the nature of the active site, and alter the catalytic rate of the enzyme complex.

Factor VIII binding sites for fIXa have been localised to amino acid residues Ser<sup>558</sup>-Gln<sup>565</sup> and Arg<sup>698</sup>-Ser<sup>710</sup> of the A<sub>2</sub> domain (O'Brien et al, 1995)(Fay et al, 1994)

and residues 1811-1818 of the A<sub>3</sub> domain (Lenting et al., 1996). Synthetic peptides corresponding to each region inhibit intrinsic tenase activity of fIXa, suggesting that both sites are required for maximal affinity of fVIIIa for fIXa (Lenting et al., 1996). Factor IXa contains at least two interaction sites for fVIIIa (Lenting et al., 1994; O'Brien et al., 1995) and equilibrium binding studies performed by Lenting and colleagues have demonstrated a high affinity surface proximal interaction involving the light chain of fIXa and the light chain (A<sub>3</sub>-C<sub>1</sub>-C<sub>2</sub> subunit) of fVIIIa, and a weak affinity surface distal site involving participation of the catalytic domain of fIXa and the heavy chain subunit (A<sub>2</sub> plus possibly A<sub>1</sub> subunit) of fVIIIa. Molecules of sufficient negative electrical charge may disrupt such interactive sites by a charge-dependent mechanism, altering the affinity of fVIIIa/IXa, and in turn, the effective enzyme concentration.

Furthermore, knowledge of the effects of individual mutations within the different regions of the fIX gene have provided insight into the function of the different domains of fIX (Giannelli et al., 1994). In particular, the importance of the first and second EGF domains is demonstrated by the detrimental effects of many mutations found in these domains (Kurachi et al., 1993). Recombinant DNA techniques have led to the production of several chimeric molecules (Lin et al., 1990; Cheung et al., 1991; Toomey et al., 1992; Zhong et al., 1994; Chang et al., 1995). Chang and colleagues made a chimeric factor IX molecule containing the EGF-1 domain from fVII, fIX<sub>VII EGF1</sub>, which possesses increased functional activity in a one-stage clotting assay when compared to recombinant wild-type fIX (Chang et al., 1997). In vitro studies



demonstrated that the increased activity is due to a higher affinity of fIX<sub>VIIIEGFI</sub> for fVIIIa, with a  $K_{d_{app}}$  one order of magnitude higher than recombinant wild-type fIXa, and when infused into a dog with haemophilia B, the chimeric fIX exhibited a greater than three-fold increase in clotting activity. Thus, disruption in critical binding sites between enzyme and cofactor by a charge-dependent effect, or alternatively, changes in conformation of the enzyme subsequently causing disruption of its interaction with the cofactor, could lead to impaired binding of substrate, reducing the catalytic efficiency of the enzyme complex.

Further evidence in favour of Scheme 'B' comes from studies using recombinant techniques and cell culture. A mutant fIX molecule with arginine at position 338 changed to alanine, possesses approximately three times greater clotting activity than wild-type fIX. The major effects of the mutation were manifest only in the presence of fVIIIa, causing a three-fold increase in  $k_{cat}$  and a two-fold decrease in  $K_m$ . The mutant fIX molecule had reduced affinity for heparin leading to the proposal that Arg<sup>338</sup> is part of an exosite that binds both fX and heparin (Chang et al., 1998). Analogous to the well-characterised exosites on thrombin, which have multiple functions including cofactor, substrate and heparin binding (Stubbs and Bode, 1993), the  $\alpha$ -helix 330-338 of fIX is close to part of the region designated exosite II in thrombin (Bode et al., 1992) which is involved in heparin binding. Using fluorescent labelling techniques, the formation of a mucopolysaccharide/fIXa complex was characterised by a stoichiometry of 1:1 with a dissociation constant  $K_{H.IXa} = 2.58 \times 10^{-7}$  M. (Jordan et al., 1980). Thus, hypersulphated LMWH may bind to an exosite(s),

causing an unfavourable interaction during the initial binding of the substrate at the active site, resulting in reduced  $k_{cat}$ . If heparin binds to extended interaction sites on the enzyme, disrupting enzyme-cofactor interaction, this model would take into account the increase in  $Kd_{app}$  seen with kinetic assays of prothrombinase. However, this does not explain the lack of increase in  $Kd_{app}$  seen with kinetic assays of intrinsic tenase.

At present, neither model 'A' nor 'B' fully explains all the observations noted in this study, and further investigations are planned to further define the mechanism of inhibition.

#### **4.4. Future Directions :**

Two models have been proposed to account for the cofactor-dependent, phospholipid-independent, noncompetitive mechanism of inhibition of the multicomponent enzymes, prothrombinase and intrinsic tenase, yet neither model entirely accounts for all the study observations. Future experiments will be performed to distinguish between these two models to more thoroughly understand the mechanism of inhibition of sLMWH on coagulation.

##### **4.4.1. in vitro Studies:**

*Determination of the Effect of sLMWH on the Enzyme-Cofactor Interaction, in the Presence of Phospholipid :* The inhibitory effect of hypersulphated LMWH on prothrombinase and intrinsic tenase activity may involve a reduction in the affinity of the enzyme-cofactor interaction. In addition to performing replicates of the assays to



determine  $K_{d_{app}}$  of fVa and fXa towards prothrombinase, and fIXa towards intrinsic tenase to aid statistical analysis of significance, the following experiments are planned:

**a. Factor VIIIa-IXa Binding :**

The binding of fVIIIa to fIXa on phospholipid membranes can be monitored using a fluorescent active site-blocked derivative of fIXa, fluorescein-thioacetyl-maleimide-FPR-IXa (F1-M-FPR-IXa), which undergoes an increase in fluorescence anisotropy when it binds fVIIIa (Barrow et al., 1994)(Duffy et al., 1992)(Mutucumarana et al., 1992). Barrow and coworkers demonstrated that the binding of fVIIIa to fIXa on phospholipid membranes was not altered in the presence of a heparin preparation which consisted of a mixture with high and low affinity for antithrombin. From these observations, Barrow and colleagues concluded that heparin does not inhibit intrinsic tenase function by interfering with the formation of the fVIIIa/IXa complex. (Barrow et al., 1994a).

A similar experiment could be performed involving titration of fVIIIa (0-100nM) into a solution of PCPS (24-50 $\mu$ M) and 20nM f-FPR-IXa, in the absence or presence of sLMWH, and monitoring the fluorescein fluorescence. In the absence of phospholipid surface, fluorescence can not be used to monitor fIXa interaction with fVIIIa alone, since no change in intensity or anisotropy is observed (Duffy et al., 1992). These experiments could also be repeated in the presence of factor X.

**b. Factor Va-fXa Binding:**

There are several published spectroscopic methods which allow the determination of the  $K_d$  values for fVa and fXa interactions with prothrombinase. The interactions of

prothrombin, fXa and fVa with phospholipid vesicles can be quantified by light scattering spectroscopy (Bloom et al., 1979). PCPS vesicles (20-50 $\mu$ M) are titrated with increasing concentrations of ligand in the absence or presence of sLMWH, and determination of light scattering intensity at 320nm after each addition. From plots of relative molecular weight vs protein/phospholipid ratio, the  $K_d$  value can be determined. The effect of sLMWH on fXa binding can also be studied by analysis of the fluorescence intensity of the fluorescent fXa derivative, dEGR-fXa (dansyl-Glu-Gly-Arg-fXa); the  $K_d$  for the interaction of fVa with dEGR.fXa on PCPS vesicles can be determined by titration of fVa (0-100nM) into a solution of 10nM dEGR-Xa and 50 $\mu$ M PCPS whilst monitoring dansyl fluorescence (Krishnaswamy, 1990).

**c. The Binding of sLMWH to Cofactors fV and fVIII, and Enzymes fIXa and fXa :**

Binding of hypersulphated LMWH to a site(s) on the enzyme would lend support to model "B". Factor IXa binds to heparin-Sepharose columns at pH 7.4 more tightly than fX or fVIII (Bloom,1979) and it has been proposed that the binding of heparin to intrinsic tenase occurs primarily via a binding site on fIXa (Brandstetter et al., 1995). It would be important to determine the presence of binding sites on fXa, in addition to factors Va and VIIIa by the same approach of affinity chromatography.

In addition, using the method of Jordan et al. (Jordan et al., 1980), a fluorescent label will be incorporated into a hypersulphated LMWH, that does not alter the functional properties of the glycosaminoglycan. The labelled sLMWH will be utilised in conjunction with fluorescence polarisation spectroscopy to monitor its binding to fIXa, fXa and other coagulation proteins, and provide direct estimates of the

stoichiometries and dissociation constants of these processes. Comparisons will be made to N-desulphated LMWH, unfractionated heparin and LMWH (Jordan et al., 1980).

#### **d. Use of Coagulation Protein Variants:**

Variant coagulation proteins, such as EGF and Gla-domainless fX (Rezaie and Esmon, 1994)(Morita and Jackson, 1986)(Sabharwal et al, 1997), chimeric fIX (Chang et al., 1997)) and fX proteins (Duffy et al.,1992b)(Cooper et al., 1997) and prethrombin-2 derivatives (Krishnaswamy and Walker, 1997), could be used in functional assays of prothrombinase and intrinsic tenase, in the presence of sLMWH, to determine if specific domains of each substrate, enzyme and cofactor may be implicated in the inhibitory mechanism.

### *2. Determination of the Effect of sLMWH on Cellular Surfaces :*

#### **The Effect of sLMWH on Prothrombinase and Intrinsic Tenase Using Activated Monocytes as a Membrane Surface:**

Mononuclear phagocytes constitute one of the major components in the cellular infiltrates that characterise atherosclerotic, neoplastic and chronic inflammatory lesions (Steinberg et al., 1991a)(Steinberg,1991b). Studying the response of monocyte procoagulants to sLMWH is of importance for the characterisation of the inhibitory effect in a biologically relevant microenvironment, and also to investigate the potential role of sLMWH in the management of hypercoagulable states associated with

atherosclerosis, inflammation and cancer (McGee et al., 1995).

Mononuclear cells will be isolated from the blood of human volunteers by centrifugation through discontinuous (Percoll) density gradients (Boyum, 1968)(English and Andersen, 1974). Monocytes will be further separated from the mononuclear cell population by centrifugation through hypertonic Percoll gradients (45% Percoll in NaCl at 9.3mg/ml) as described previously (Boyum, 1968)(McGee et al., 1995)(McGee and Li, 1991). Contaminating platelets will be removed using differential centrifugation, and the cell populations isolated for use in kinetic experiments will be determined by morphologic criteria using Wright's stain, and also by flow cytometry. Assays of intrinsic tenase and prothrombinase will be performed using continuous techniques as described in "Experimental Methods" (Section 2.2.2). Experiments carried out to assess the effects of different chondroitin sulphates (CS) and heparan sulphate on intrinsic tenase activity using activated monocytes as a surface demonstrated that CS with 4,6-di-O-sulphated N-acetylgalactosamine glycosaminoglycan structures inhibited intrinsic tenase activity (McGee et al., 1995). It is anticipated that sLMWH will also inhibit intrinsic tenase and prothrombinase on the monocyte surface.

#### **4.4.2. In Vivo Studies : Animal Models**

Hypersulphated LMWH has potential advantages over heparin and LMWH by its potent ability to inhibit phospholipid-bound fXa and fIXa. A rat model of acute arterial thrombosis has been designed (Klement et al., 1992) in which the distal aorta

undergoes balloon injury. Tourniquets are used to isolate the damaged segment, and thrombus produced by injecting 200µl recalcified rat blood with  $^{125}\text{I}$ -labelled rabbit fibrinogen. Two aliquots of blood will be recalcified in tubes, and following incubation for 30 minutes at 37°C, the mean of the weight and radioactivity will be used as a baseline of the initial weight and radioactivity of clots in the aorta. After allowing clot in the aorta to age for 30 minutes, the tourniquet is removed, and external constriction is applied around the aorta to reduce the blood flow. Animals will be randomly assigned to receive a bolus and a 60 minute infusion of sLMWH versus LMWH versus heparin, in concentrations producing equivalent anti-fXa activity. Blood will then be collected to measure the APTT and anti-Xa activity. After 60 minutes, animals will be sacrificed and the clots in the aorta removed, weighed and counted. By subtracting values from weight and radioactivity of the clots at the outset, the extent of clot accretion can be determined. This model shows evidence of considerable clot accretion in animals given saline. It is anticipated that sLMWH will be a potent antithrombotic in this model given its unique mechanism of action.

#### **4.5. Summary and Perspectives**

From the results of this study, I conclude that :-

1. In buffer systems, hypersulphated LMWH directly inhibits not only intrinsic tenase, but also prothrombinase at concentrations used in therapeutic settings.
2. The mechanism of inhibition is charge-dependent; by causing an increase in the negative charge, progressive hypersulphation of LMWH increases the direct inhibitory effect towards the activities of intrinsic tenase and prothrombinase.
3. Results from buffer systems have also been supported by plasma-based assays, which show that hypersulphated LMWH has an antithrombin-independent inhibitory effect on both enzyme complexes in plasma.
4. The mechanism of inhibition is cofactor-dependent and phospholipid-independent.
5. Data from kinetic studies demonstrate that hypersulphated LMWH inhibits intrinsic tenase and prothrombinase in a noncompetitive manner.

From kinetic data, I have proposed two models, although data is at present insufficient to support or refute either model completely. I have also suggested further studies to help elucidate the mechanism of inhibition further.

By directly inhibiting prothrombinase and intrinsic tenase activity, hypersulphated LMWH can be added to a growing list of direct enzyme complex inhibitors, which include active site-blocked fXa and fIXa (Benedict et al., 1991; Wong et al., 1997), fIXa antibodies (Feuerstein et al., 1997) and active-site directed fXa inhibitors (Tuszynski et al., 1987; Vlasuk, 1993; Cappello et al., 1995; Taniuchi et al.,

1998). Hypersulphated LMWH may have potential clinical advantages over these agents by inhibiting both fXa and thrombin generation simultaneously, with selectively greater inhibition of fXa generation by intrinsic tenase.

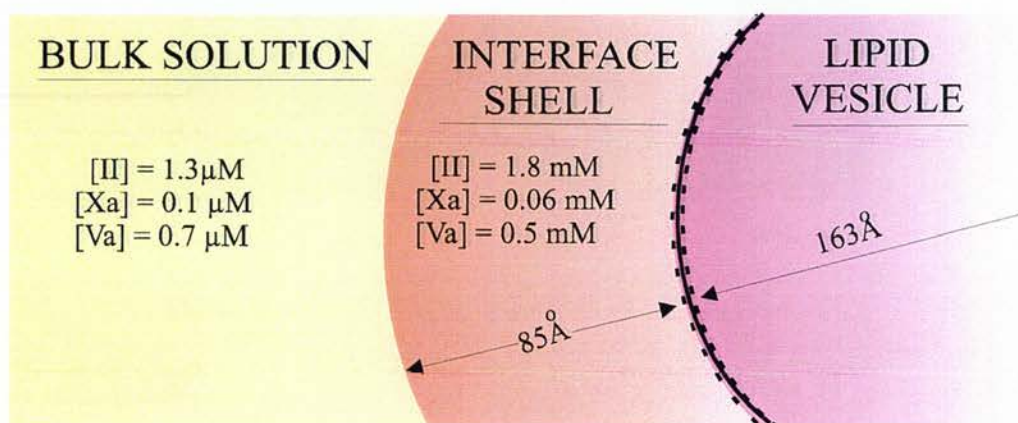
Additionally, hypersulphated LMWHs may prove to be useful as scientific tools in the laboratory; for these reasons, it is important that as much as possible is understood about the mechanism of inhibition of this novel series of anticoagulants.



## Appendix (i)

Surface-mediated Enzymatic Reaction : "CLOTSPEED MODEL" to account for the surface-enhanced rate of thrombin formation by the prothrombinase complex.

From the known binding parameters, the distribution of substrate and enzymatic components between bulk solution and vesicle can be calculated for any concentration of substrate, enzyme and phospholipid. In a physiological setting, most of the substrate (97%) is in solution; the remainder is associated with the vesicle surface. The bound "substrate" contributes toward saturation of the vesicle surface. Formal local concentrations of enzyme and substrate have been determined; using light scattering experiments, it has been shown that when proteins bind to the phospholipid surface, the hydrodynamic radius of the phospholipid protein complex exceeds that of the phospholipid vesicle. ie. a shell (interface shell) develops around a phospholipid vesicle, and within this shell, formal local concentrations of enzyme and substrate can be calculated in the region of the vesicle surface. This model accounts for the remarkable rate enhancement seen with the assembly of the complex. the phospholipid surface is "passive" and provides a locus for the condensation of enzyme, substrate and cofactor. The co-concentration of substrate and enzyme increases the turnover number of substrate-enzyme complex by a factor of 3100. These two effects are multiplicative and the overall rate is increased by 280,000.



Schematic diagram showing the distribution of substrate, fXa and fVa between the bulk solution and the hypothetical "interface shell" surrounding the vesicles. Local and bulk formal protein concentrations are indicated. (Adapted from Nesheim, 1984)

(Nesheim, Tracy, et al. 1984), (Nesheim, 1984)



## Appendix (ii)

Inorganic Phosphate Assay (Ames, 1966)

Principle : The phosphomolybdate complex is reduced by ascorbic acid resulting in a colour change.

Reagents : (a) Ascorbic acid 10%

(b) 0.42% Ammonium molybdate.4 H<sub>2</sub>O in 1M H<sub>2</sub>SO<sub>4</sub>

"Mix" = 1 part of (a) to 6 parts of (b)

Procedure : 0.7 ml of the "mix" was added to 0.3 ml of phosphate solution in a small test tube and incubated for 1 hour at 37°C ( or 20 minutes at 45°C). At 820nm, 0.01  $\mu$ M inorganic phosphate results in an absorbancy of 0.26, and the colour is stable for several hours. A standard curve can be made as readings are proportional to phosphate concentrations.

All glassware must be scrupulously clean (free of phosphate), and tubes were acid-washed and rinsed with distilled water before use.

Ashing Procedure for Total Phosphate

Principle : The sample of organic phosphate and a drop of magnesium nitrate solution are taken to dryness in a small test tube by shaking the tube in a Bunsen burner flame. This procedure completely ashes the organic phosphates, and the procedure can then be coupled with the sensitive inorganic phosphate method described above.

Reagents : 10% Mg(NO<sub>3</sub>)<sub>2</sub>.6H<sub>2</sub>O in 95% alcohol

0.5M HCl

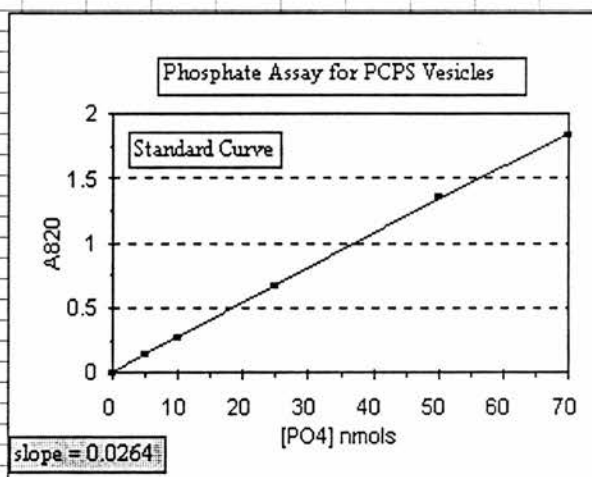
"mix" described in assay for inorganic phosphate

Procedure : 30 $\mu$ l of magnesium nitrate solution was added to 0.01 - 0.1 ml of phosphate sample containing up to 70nmol of phosphate. The material was taken to dryness by shaking in a Bunsen burner until the brown fumes disappeared. The tubes were then cooled to room temperature and 0.3ml of 0.5M HCl added; the tube was capped with a marble and heated in a boiling water bath for 15 minutes to hydrolyse to phosphate any pyrophosphate formed in the ashing. After the tubes were cooled, 0.7ml of "mix" was added, and the tubes were incubated as in the inorganic phosphate assay. Absorbance was read at 820nm.

Phosphate Assay for Vesicles Made Sept 26/96

PO <sub>4</sub> (nmols)	A820
0	0
5	0.144
10	0.274
25	0.672
50	1.352
70	1.843

Sample	Vol( $\mu$ l)	A820	nmol P	[PCPS]mM
1a	5	0.266	9.704	1.9
1b	10	0.542	20.15	2



### Appendix (iii)

#### Determination of the Turnover Number for Intrinsic Tenase

##### *(a) Determination of the Specific Activity of fXa towards the Chromogenic Substrate, N-methoxycarbonyl-D-norleucyl-L-arginine-4-nitranilide-acetate (ChZX)*

The assay was performed as follows:

10 $\mu$ l of 0-1000nM fXa was added to 90 $\mu$ l 400 $\mu$ M ChZX, and the change in OD monitored at 405nm for 5 minutes at RT. The slope of the graph [Xa] vs change in OD/time = 7.87 mOD/min/nM Xa, and 1nM fXa = 0.00787 OD/min.

##### *(b) Effect of Unfractionated Heparin on the Hydrolysis of ChZX by fXa*

The assay was performed as follows:

80 $\mu$ l of 400 $\mu$ M ChZX was added to 10 $\mu$ l 100nM fXa in the presence of a final concentration of 1-1000 $\mu$ g/ml unfractionated heparin. The change in optical density was monitored at 405nm for 5 minutes at RT.

No significant effect on the rate of hydrolysis was noted.

##### *(c) Effect of Hypersulphated LMWH on the Turnover Number for Intrinsic Tenase*

The catalytic rate of intrinsic tenase was determined by monitoring the rate of fXa generation using the chromogenic substrate, Chromozym X, in a continuous assay, using the following final reactant conditions (Chang et al., 1997):-

fVIII/fVIIIa 0.48nM fX 192nM CaCl<sub>2</sub> 8mM PCPS 41 $\mu$ M fIXa<sub>1</sub> 2.5nM

ChZX 500 $\mu$ M

Albumin-free fVIII was activated by 2.5nM thrombin for exactly five minutes, and was added to a stock solution containing calcium, fIXa and PCPS vesicles. 102 $\mu$ l of this stock solution was aliquoted into a flat-bottomed 96-well microtitre plate. A series of assays were performed simultaneously in the presence of increasing concentrations of hypersulphated LMWH, and with a control well using Tris-PEG 0.1% buffer in place of glycosaminoglycan.

Using a multichannel pipette, a final concentration of ChZX was added to the series of wells, and fX then added to initiate the reaction. A final volume of 200 $\mu$ l was used. The order of addition of reactants was critical.

Initial rates of chromogenic substrate hydrolysis were followed kinetically by monitoring the change in optical density every 11 seconds on a Spectra-max 340 microplate reader, using the manufacturer's Softmax software. All reactions were monitored at RT for five minutes, at 405nm.

#### Data Analysis

Data was analysed as in Section 2.2.10

#### **Determination of the Michaelis Constant (Km) for Intrinsic Tenase, at Saturating Concentration of Phospholipid Vesicles, in the Absence and Presence of Hypersulphated LMWH.**

The Km, a measure of the affinity of the fVIIIa/fIXa complex for the substrate, fX, was determined by measuring fXa generation

in the reaction that contained :-

**fVIIIa 0.48nM fIXa 2.5nM CaCl<sub>2</sub> 5mM PCPS 41μM ChZX 500μM**

**fX 0-384nM**

A series of nine dilutions of fX were made giving a concentration range of 1-400nM.

0.48nM fVIII was activated with 1nM thrombin for exactly five minutes. 48μl of this solution was added to a stock containing 54μl CaCl<sub>2</sub>, fIXa<sub>p</sub> and PCPS vesicles in a microtitre well plate, to which 50μl ChzX was added. 50μl of fX was added to initiate the generation of fIXa, in a final reaction volume of 200μl. The procedure was simultaneously repeated in the presence of 0.5 and 0.25μg/ml V20S. Assays were performed in duplicate.

Changes in optical density at 405nm were monitored for 5 minutes on a Spectra-max 340 micro-plate reader, at room temperature, and converted to the fIXa generation rate.

#### Data Analysis

See Section 2.2.11

#### **Determination of the Apparent K<sub>d</sub><sub>IXa</sub> for Intrinsic Tenase at Saturating Concentration of Phospholipid Vesicles, by Increasing the Enzyme Concentration in the Absence and Presence of Hypersulphated Heparin.**

The apparent K<sub>d</sub> of fIXa for intrinsic tenase, at saturating concentrations of PCPS vesicles, (a measurement of the affinity of fIXa towards the intrinsic tenase complex), was determined by measuring the rate of fIXa generation in the reaction that contained :-

**fVIIIa 0.48nM fX192nM PCPS vesicles 41μM CaCl<sub>2</sub> 5mM ChZX 500μM**

**fIXa<sub>p</sub> 0-50nM**

The assay was performed as follows :

A series of ten dilutions of 50μl fIXa were made in Eppendorf tubes, to which CaCl<sub>2</sub> and PCPS vesicles were added. 48μl fVIII was then activated using 1nM thrombin for exactly 5 minutes at room temperature. The fIXa/calcium/PCPS mixture was then aliquoted into a series of microtitre wells, to which the fVIII mixture was added. 50μl ChZX was then added to the ten wells simultaneously using a multichannel pipette, and 50μl fX was subsequently added to initiate the reaction. The procedure was simultaneously carried out in the presence of 0.25μg/ml V20S. Assays were performed in duplicate.

Changes in optical density at 405nm were monitored for 5 minutes on a Spectra-max 340 micro-plate reader, at room temperature, and converted to the fIXa generation rate.

#### Data Analysis See section 2.2.12

**Appendix (iv)**

Tyrode's modified buffer (TMB)(pH 7.5): (Yuan, Y et al., 1996)

	100mM NaCl		
	0.32% AC	137mM NaCl	
<u>Reagent</u>	<u>500ml</u>	<u>250ml</u>	250ml
0.5% BSA (bovine serum albumin)	2.5g		
12mM NaHCO <sub>3</sub> (84.01)	0.5g		
0.32mM NaH <sub>2</sub> PO <sub>4</sub> (137.99)	0.22g		
10mM Hepes (238.3)	1.19g		
Split into 2 x 250ml and add the following			
NaCl (58.44)		1.46g	2g
0.32% NaCitrate		0.8g	

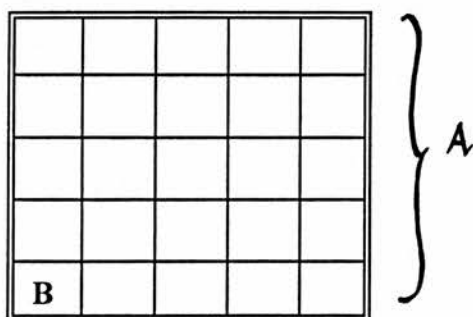
To each 10 ml of TMB used, 10 $\mu$ l of 5mM stock PGE<sub>1</sub> was added, giving a final PGE<sub>1</sub> of 5 $\mu$ M.

## Appendix (v)

### Procedure for counting cells in haemocytometer

50µl of cell suspension was added to 50µl trypan blue and mixed. 10µl of this mixture was placed on a haemocytometer counting slide and the cells counted as indicated.

Note: Any cells that stain blue in the presence of trypan blue have died.



If cells in one very large square A are counted:

$$\# \text{ cells in A} \times \text{dil factor} \times 10^4 = \# \text{ cells /ml}$$

If cells in one square (B) are counted :

$$\# \text{ cells in B} \times 25 \times \text{dil factor} \times 10^4 = \text{cells/}$$

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